Copy for the Elected Office (EO/US) PCT/US00/16486

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

MONACO, Daniel, A.
Drinker Biddle & Reath LLP
One Logan Square
18th and Cherry Streets
Philadelphia, PA 19103-6996
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)
27 juillet 2001 (27.07.01)

Applicant's or agent's file reference
6056-268 PC

IMPORTANT NOTIFICATION

International application No.
PCT/US00/16486

International filing date (day/month/year)
15 juin 2000 (15.06.00)

1. The following indications appeared on record concerning:
☐ the applicant ☐ the inventor ☒ the agent ☐ the common representative

Name and Address
MONACO, Daniel, A.
Seidel, Gonda, Lavorgna & Monaco, P.C.
Suite 1800
Two Penn Center Plaza
Philadelphia, PA 19102
United States of America

State of Nationality:
State of Residence:
Telephone No.: (215) 568-8383
Facsimile No.: (215) 568-5549
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:
☐ the person ☐ the name ☒ the address ☐ the nationality ☐ the residence

Name and Address
MONACO, Daniel, A.
Drinker Biddle & Reath LLP
One Logan Square
18th and Cherry Streets
Philadelphia, PA 19103-6996
United States of America

State of Nationality:
State of Residence:
Telephone No.: (215) 988-3312
Facsimile No.: (215) 988-2757
Teleprinter No.:

3. Further observations, if necessary:
Change in agent's address.

4. A copy of this notification has been sent to:
☒ the receiving Office
☐ the International Searching Authority
☒ the International Preliminary Examining Authority
☒ the designated Offices concerned
☒ the elected Offices concerned
☐ other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
H. Zhou
Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

004179012

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 05 July 2001 (05.07.01)	
International application No. PCT/US00/16486	**Applicant's or agent's file reference** 6056-268 PC
International filing date (day/month/year) 15 June 2000 (15.06.00)	**Priority date** (day/month/year) 16 June 1999 (16.06.99)
Applicant REDDY, E., Premkumar et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   11 January 2001 (11.01.01)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

H. Zhou

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
6056-268 PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/16486	15 JUNE 2000	16 JUNE 1999
International Patent Classification (IPC) or national classification and IPC IPC(7): C12Q 1/26, 1/28; C12N 9/00, 9/02 and US Cl.: 435/7.2, 7.21, 25, 28, 183, 189		
Applicant TEMPLE UNIVERSITY- OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☐ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☐ Certain defects in the international application

   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
11 JANUARY 2001	14 AUGUST 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MANJUNATH RAO Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/16486

I. Basis of the report

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-22 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 23-25 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawings:
pages 1-7 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages 1-2 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

[X] contained in the international application in printed form.

[X] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/16486

V. Reasoned statement under Article 35(2) with regard to n velty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	2-14	YES
	Claims	1	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-14	NO
Industrial Applicability (IA)	Claims	1-14	YES
	Claims	NONE	NO

2. citations and explanations (Rule 70.7)

Claim 1 lacks novelty under PCT Article 33(2) as being anticipated by Brideau et al.

Claim 1 is drawn to a method of screening a test substance for COX-2 inhibitory activity comprising contacting the test substance with indicator cells which constitutively express endogenous COX-2 or inducibly express endogenous COX-2 and determining the level of proliferation of the indicator cells or by determining one or more prostaglandins in the presence and absence of test substance or determining the production of arachidonic acid. Brideau et al. disclose an identical assay to evaluate or screen an inhibitor for COX-2 which involves the determination of the prostaglandins in the presence and absence of the inhibitory agent (see page 69, Materials and Methods and page 70 and figures 2-4). Therefore Brideau et al. anticipate claim 1 as written.

Claims 1-14 lack an inventive step under PCT Article 33(3) as being obvious over Xu et al. in view of Brideau et al. Claims 1-14 are drawn to methods of screening a test substance for COX-2 inhibitory activity comprising 1)contacting the test substance with indicator cells which constitutively express endogenous COX-2 or inducibly express endogenous COX-2 and determining the level of proliferation of the indicator cells or by determining one or more prostaglandins in the presence and absence of test substance or determining the production of arachidonic acid (claim 1) or 2)a method of screening a test substance for COX-2 inhibitory activity comprising contacting the test substance with indicator cells which express a GTPase-deficient mutant of the alpha-subunit of protein G12, which mutant alpha-subunit has the capacity to induce the production of arachidonic acid and determining the level of proliferation of the indicator cells (claim 2) or 3)a method of screening a test substance for COX-2 inhibitory activity comprising contacting the test substance with indicator cells which express a GTPase-deficient mutant of the alpha-subunit of protein G12, which mutant alpha-subunit has the capacity to induce the production of arachidonic acid and determining the level of one or more prostaglandins produced by (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
the indicator cells (claim 6) or 4) a method of screening a test substance for COX-2 inhibitory activity comprising contacting the test substance with indicator cells which express a GTPase-deficient mutant of the alpha-subunit of protein G12, which mutant alpha-subunit has the capacity to induce the production of arachidonic acid and determining the level of arachidonic acid produced by the indicator cells (claim 11).

Brideau et al. disclose an assay to evaluate or screen an inhibitor for COX-2 which involves the determination of the prostaglandins in the presence and absence of the inhibitory agent (see page 69, Materials and Methods and page 70, figures 2-4). However, the reference does not teach the existence of the GTPase-deficient mutant of the alpha-subunit of protein G12, which mutant alpha-subunit has the capacity to induce the production of arachidonic acid or the capacity to induce proliferation of cells.

Xu et al. teach a mutant alpha subunit of G12 protein which potentiates the eicosanoid pathway and is highly transforming in NIH 3T3 cells (see Introduction page 6741, page 6745, Discussion columns 1 and 2). The reference also teaches that cells transfected with alpha1-2-QL exhibited a remarkable increase in release of arachidonic acid in response to serum. The reference also teaches that once arachidonic acid is released from phospholipids, it is oxidized by one of three different enzymes generating leukotrienes, lipoxins or prostaglandins. Thus the above reference teaches that the alpha-12-QL mutant has the capacity to induce proliferation, arachidonic acid and prostaglandins which can be monitored by an assay.

Thus, combining the references of Brideau et al. and Xu et al. it would have been obvious to one of ordinary skill in the art to develop a method of screening a test substance for COX-2 activity. One of ordinary skill in the art would have been motivated to do so as Brideau et al. teach that selective COX-2 inhibitors may comprise a new class of therapeutic agents with similar anti-inflammatory effects to conventional NSAIDs, but with a substantially improved side effect profile. One of ordinary skill in the art would have a reasonable expectation of success since Xu et al. teach a mutant alpha subunit of G12 protein which potentiates the eicosanoid pathway and is highly transforming in NIH 3T3 cells and Brideau et al. teach assays for screening inhibitors of COX-1 and COX-2. Therefore the above invention would have been *prima facie* obvious to one of ordinary skill in the art.

---------- NEW CITATIONS ----------
NONE

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: DANIEL A. MONACO
SEIDEL, GONDA, LAVORGNA & MONACO, P.C.
SUITE 1800
TWO PENN CENTER PLAZA
PHILADELPHIA PA 19102

NOV 2 2 2000

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing *(day/month/year)* 17 NOV 2000
Applicant's or agent's file reference 6056-268 PC	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US00/16486	International filing date *(day/month/year)* 15 JUNE 2000
Applicant TEMPLE UNIVERSITY- OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION	

1. [X] The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 *bis* 1 and 90 *bis* 3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MANJUNATH RAO
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/220 (July 1998)*

(See notes on accompanying sheet)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 6056-268 PC	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US00/16486	International filing date *(day/month/year)* 15 JUNE 2000	(Earliest) Priority Date *(day/month/year)* 16 JUNE 1999
Applicant TEMPLE UNIVERSITY- OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   [ ] contained in the international application in written form.
   [ ] filed together with the international application in computer readable form.
   [ ] furnished subsequently to this Authority in written form.
   [ ] furnished subsequently to this Authority in computer readable form.
   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. [ ] **Certain claims were found unsearchable** (See Box I).
3. [ ] **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:
5. With regard to the **abstract**,
   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the **drawings** to be published with the abstract is Figure No. ____
   [ ] as suggested by the applicant.
   [ ] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.
   [X] None of the figures.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: DANIEL A. MONACO
SEIDEL, GONDA, LAVORGNA & MONACO, P.C.
SUITE 1800
TWO PENN CENTER PLAZA
PHILADELPHIA PA 19102

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)* 29 AUG 2001	
Applicant's or agent's file reference 6056-268 PC	IMPORTANT NOTIFICATION
International application No. PCT/US00/16486	International filing date *(day/month/year)* 15 JUNE 2000
Priority Date *(day/month/year)* 16 JUNE 1999	
Applicant TEMPLE UNIVERSITY- OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION	

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer MANJUNATH RAO
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/IPEA/416 (July 1992)*

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 December 2000 (21.12.2000)

PCT

(10) International Publication Number
WO 00/77245 A1

(51) International Patent Classification[7]: C12Q 1/26, 1/28, C12N 9/00, 9/02

(21) International Application Number: PCT/US00/16486

(22) International Filing Date: 15 June 2000 (15.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/139,569 16 June 1999 (16.06.1999) US

(71) Applicant *(for all designated States except US)*: **TEMPLE UNIVERSITY - OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION** [US/US]; Broad Street And Montgomery Avenue, Philadelphia, PA 19122 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **REDDY, E., Premkumar** [US/US]; 547 Atterbury Road, Villanova, PA 19085 (US). **REDDY, M.V., Ramana** [IN/US]; 354B Beverly Boulevard, Upper Darby, PA 19082-4504 (US). **DHANASEKARAN, N.** [US/US]; 103 Drakes Drum Drive, Bryn Mawr, PA 19010-1126 (US).

(74) Agent: **MONACO, Daniel, A.**; Drinker Biddle & Reath LLP, One Logan Square, 18th and Cherry Streets, Philadelphia, PA 19103-6996 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(48) Date of publication of this corrected version:
20 June 2002

(15) Information about Correction:
see PCT Gazette No. 25/2002 of 20 June 2002, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/77245 A1

(54) Title: CELL-BASED ASSAY FOR SCREENING COX-2 INHIBITORS

(57) Abstract: A cell-based assay is provided for screening substances for the capacity to inhibit COX-2 activity. The assay utilizes as a screening reagent a cell line which has been engineered to achieve constitutive or inducible expression of COX-2. In one embodiment, the cells express a GTPase-deficient, constitutively-activated mutant form of the α-subunit of protein G12 which induces the production of arachidonic acid and COX-2. Decreased cell proliferation in the presence of test substance indicates that the substance has COX-2 inhibitory activity.

CELL-BASED ASSAY FOR SCREENING COX-2 INHIBITORS

Cross-Reference to Related Application

The benefit of the filing date of U.S. provisional patent application Ser. No. 60/139,569 filed June 16, 1999 is hereby claimed pursuant to 35 U.S.C. 119(e). The entire disclosure of the aforesaid provisional application is incorporated herein by reference.

Reference to Government Grant

The invention described herein was supported in part by National Institutes of Health grant GM49897. The U.S. government has certain rights in the invention.

Field of the Invention

The invention relates to a method for the identification of compounds having biological activity. In particular, the invention relates to the identification of compounds which inhibit the activity of cycloxygenase-2.

Background of the Invention

Heterotrimeric G Protein

Heterotrimeric guanine nucleotide-binding regulatory proteins ("heterotrimeric G proteins") that transduce signals from cell surface receptors to intracellular effectors are composed of α, β and γ subunits. Gα subunits are grouped based on amino acid homology into four subfamilies: G_s, G_i, G_q and G_{12} (Strathman and Simon, *Proc. Natl. Acad.*

Sci. (USA) 1991; 88:5582-5582). The α subunits possess intrinsic GTPase activity and belong to a much larger group of GTPases which share structural elements. In all of these GTPases, a cycle of guanine nucleotide exchange and hydrolysis enables the protein to exist in two distinct states. The cycle allows G proteins to transiently relay signals from cell-surface receptors to intracellular effectors. Upon interaction with the appropriate agonist, the receptor serves to accelerate the exchange of GDP for GTP on the G protein α subunit. The exchange is believed to be accompanied by dissociation of the α and β-γ subunits, allowing α (and in some cases β-γ) to interact with effectors. The intrinsic GTPase activity terminates the signal, returning the α subunit to its basal GDP-bound state. Studies have suggested that the G_{12} members α_{12} and α_{13} regulate signaling pathways involved in controlling cell growth and differentiation. *See* Vara Prasad *et al.*, *J. Biol. Chem.* 1995, 270:18655-18659.

The full-length cDNAs encoding mouse Gα12 and Gα13, and the encoded translation products, are disclosed in Strathman and Simon, *supra*, the entire disclosure of which is incorporated herein by reference. The sequences have been deposited in the GenBank data base by the authors, accession nos. M63659 (Gα12) and M63660 (Gα13). Those GenBank records are incorporated herein by reference. The human $G\alpha_{12}$ cDNA has also been cloned (Chan *et al.*, *Mol. Cell. Biol.* 1993; 13:762-768, incorporated herein by reference).

When constitutively activated, GTP-binding proteins can induce neoplastic transformation. *See* Xu *et al.*, *Proc. Natl. Acad. Sci. (USA)* 1993: 90:6741-6745, the entire disclosure of which is incorporated herein by reference. Overexpression of wild-type $G\alpha_{12}$ in NIH 3T3 cells is weakly transforming; a GTPase-deficient mutant of $G\alpha_{12}$ ($G\alpha_{12}$Q229L) behaves as a potent oncogene and is highly transforming in NIH 3T3 cells (Xu *et al.*, *supra*). It has been found that $G\alpha_{12}$-transfected cells exhibit a remarkably increased level of arachidonic acid in response to serum, and this effect is observed in cells transfected with either wild-type or activated mutant $G\alpha_{12}$

(Xu *et al.*, *supra*).

The $G\alpha_{12}$Q229L mutant results from insertion of a leucine residue at position 229 in lieu of glutamine in the wild-type $G\alpha_{12}$ protein. The mutation occurs in a highly conserved region in G proteins which is involved in binding and GTPase activity. The Q229L mutation results in a GTPase-deficient form of $G\alpha_{12}$. The mutation blocks GTPase activity so that the α subunit binds GTP and is constitutively active (Xu *et al.*, *supra*; Dhanasekaran *et al.*, *J. Biol. Chem.* 1993, 269:11802-11806; Jian *et al.*, *FEBS Lett.* 1993; 330:319-322; Vara Prasad *et al.*, *Oncogene* 1994, 9:2425-2429; Voyno-Yasenetskaya *et al.*, *Oncogene* 1994; 9:2559-2565).

COX-2

Cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) are the enzymes which convert arachidonic acid into prostaglandins. COX-1 is ubiquitously expressed and involved in cellular "housekeeping" functions of various tissues and organs. In contrast, COX-2 expression is rapidly induced in diverse cell types by different growth factors, mitogens, tumor promoters, and physiological stress stimuli. Transcriptional induction of COX-2 has been shown to be involved in different pathological conditions such as inflammation, pain, and fever. It has also been shown that the anti-inflammatory effects of aspirin and ibuprofen is through their inhibitory effect, albeit non-specific inhibitory effect, on COX-2. Furthermore, persistent activation of COX-2 has been shown to be associated with oncogenesis as well as the invasive potential of tumor cells.

Inhibitors of COX-2 are useful as therapeutics. See, for example, the discussion of the advantages of selective COX-2 inhibition set forth in U.S. Pat. 5,604,253.

What is needed is a simple, sensitive and rapid screening method for determining the COX-2 inhibitory activity of therapeutic candidates.

Summary of the Invention

It is an object of the invention to provide a screening method to determine the COX-2 inhibitory activity of chemical compounds.

It is an object of the invention to provide a screening method for COX-2 inhibition which relies on cells which respond to the presence of COX-2 inhibitor by reducing proliferation in comparison to cells of the same type which are not contacted with COX-2 inhibitor.

It is an object of the invention to provide a screening method for COX-2 inhibition which relies on cells which respond to the presence of COX-2 inhibitor by increasing production of one or more prostaglandins in comparison to cells of the same type which are not contacted with COX-2 inhibitor.

It is an object of the invention to provide a screening method for COX-2 inhibition which relies on cells which respond to the presence of COX-2 inhibitor by accumulating arachidonic acid in comparison to cells of the same type which are not contacted with COX-2 inhibitor.

A method for screening a test substance for COX-2 inhibitory activity is provided. The test substance is contacted with indicator cells which constitutively or inducibly express endogenous COX-2. The level of proliferation of the indicator cells is determined in the presence and absence of the test substance. A decreased level of proliferation of the indicator cells in the presence of the test substance indicates that the test substance has COX-2 inhibitory activity.

According to another method for screening a test substance for COX-2 inhibitory activity, the test substance is contacted with indicator cells which constitutively or inducibly express COX-2, and the level of one or more prostaglandins produced by the indicator cells is determined in the presence or absence of the test substance. A decreased level of one or more prostaglandins produced by the indicator cells in the presence of a test substance indicates that the test substance has COX-2 inhibitory

activity.

According to another method for screening a test substance for COX-2 inhibitory activity, the test substance is contacted with indicator cells which constitutively or inducibly express COX-2, and the level of arachidonic acid provided by the indicator cells is determined in the presence or absence of the test substance. An increased level of arachidonic acid provided by the indicator cells in the presence of a test substance indicates that the test substance has COX-2 inhibitory activity.

According to preferred embodiments of the invention, indicator cells constitutively or inducibly express endogenous COX-2. According to other preferred embodiments of the invention, the indicator cells express a GTPase-deficient mutant form of the α-subunit of protein G12. The mutant α-subunit has the capacity to induce the production of arachidonic acid and COX-2 in the indicator cells.

According to one embodiment of the invention, the G12 protein α-subunit mutant comprises the Q229L mutation.

The level of indicator cell proliferation is conveniently determined by an assay for DNA synthesis by the indicator cells. According to one embodiment, the DNA synthesis assay comprises assaying tritium-labeled thymidine uptake by the indicator cells.

The level of indicator cell production of one or more prostaglandins is conveniently determined with resort to any assay which is capable of quantifying, at least in relative terms as compared to control cells, the level of production of such prostaglandins. According to a preferred embodiment, the prostaglandin assay method comprises an immunoassay. According to a more preferred embodiment, the immunoassay comprises a competitive immunoassay for a prostaglandin.

The level of arachidonic acid in the indicator cells, the components thereof, or released into the medium surrounding the cells, is conveniently determined with resort to any assay which is capable of quantifying, at least in relative terms as compared to control cells, the level of arachidonic acid.

One such method is a radiolabeling assay. According to one embodiment, the assay comprises assaying release of tritiated arachidonic acid by the indicator cells.

Description of the Figures

Fig. 1 is a schematic representation of the mechanism by which COX-2 plays a role in proliferation of cells expressing the GTPase-deficient $G\alpha_{12}QL$ mutant.

Fig. 2A is a Northern blot of analysis of RNA from IPTG-inducible $G\alpha_{12}QL$-NIH3T3 cells stimulated with 1 mM IPTG for 0, 0.5, 1, 3 and 6 hours. Cells were probed with DNA encoding $G\alpha_{12}QL$, COX-2 or GAPDH.

Fig. 2B is similar to Fig. 2A, except that the analysis was carried out on control cells. The control cells were NIH3T3 cells transfected with vector lacking the $G\alpha_{12}QL$ insert.

Fig. 3 is Western blot analysis of cell lysates probed for COX-2 expression. The lysates were prepared from $G\alpha_{12}QL$-NIH3T3 cells (lanes 3 and 6), control NIH3T3 cells (lanes 1 and 4) and $G\alpha_{12}WT$-NIH3T3 cells expressing wild type $G\alpha_{12}$ (lanes 2 and 5).

Fig. 4 is a graph of [^{3}H]-thymidine incorporation as an index of DNA synthesis in NIH3T3 cells transfected with $G\alpha_{12}QL$ DNA ($G\alpha_{12}QL$-NIH3T3) versus control NIH3T3 cells transfected with vector alone (pcDNA3-NIH3T3). Values represent the mean ±S.E. from a triplicate determination.

Fig. 5 is a graph of prostaglandin release from NIH3T3 cells as a function of stable transfection with $G\alpha_{12}QL$ expression vectors and pcDNA3. Values represent the mean ±S.E. from a triplicate determination.

Fig. 6A is a graph of [^{3}H]-arachidonic acid release from $G\alpha_{12}QL$ expression vector or pcDNA3 transfected NIH3T3 cells. Quiescent, stably transfected cells were stimulated with serum and [^{3}H]-arachidonic acid release was followed as a function of time. Values represent the mean ±S.E. from a triplicate determination.

Fig. 6B is a graph of [^{3}H]-arachidonic acid release from pcDNA3, *ras*,

$G\alpha_{12}QL$, and $G\alpha_{13}QL$ expression vector stably transfected NIH3T3 cells. Quiescent, transfected cells were stimulated with serum and [^{3}H]-arachidonic acid release was measured after 10 minutes. Values represent the mean ±S.E. from a quadruplicate determination.

Detailed Description of the Invention

A cell based bioassay system is provided for screening compounds for COX-2 inhibitory activity by assaying the growth-inhibitory property of the compounds when exposed to COX-2 growth-driven target cells. The target cells are cells which are capable of growing in culture but which have been engineered to constitutively or inducibly express COX-2, preferably endogenous COX-2.

By "expression" with respect to COX-2 is meant the production of active COX-2 enzyme by the cell.

By "endogenous" with respect to COX-2 is meant DNA sequences corresponding to the native, COX-2 gene locus, its variants, or derivatives present in the cells of the organism from which a given indicator cell line was derived.

By "constitutively express" with respect to COX-2 is meant that biologically active COX-2 protein is present continually in a cell and does not appear to be subject to quantitative regulation.

By "inducibly express" with respect to COX-2 is meant that the COX-2 gene is activated in response to a specific stimulus, such as the presence of a specific small molecule inducer, to produce biologically active COX-2 protein. The gene is not activated, or activated only at a much lower level, in the absence of the specific stimulus. Inducible expression of the COX-2 gene may be controlled directly to achieve inducible expression, such as by an inducible promoter operably linked to a COX-2 gene in an expression construct. Alternatively, inducible expression of the COX-2 gene may be controlled indirectly, such as by an inducible promoter operably linked to a second gene other than the COX-2 gene. The inducible expression of the

second gene in turn governs the expression of COX-2.

COX-2 is activated by several different pathways in the cell (Vane *et al.*, *Annu. Rev. Pharmacol. Toxicol.* 1998; 38:97-120, incorporated herein by reference). Each pathway is a target for manipulation of COX-2 gene expression to achieve either constitutive or inducible expression of that gene. Cell lines which express endogenous COX-2 and produce prostaglandins may be appropriate indicator cells for screening for COX-2 inhibitors. An example of such a cell line is the MDA-MB-231 human breast cancer cell line (ATCC number HTB-26; Liu and Rose, *Cancer Res.* 1996; 56:5125-5127, incorporated herein by reference).

According to one embodiment, the indicator cells express a GTPase-deficient, constitutively-activated mutant form of the α-subunit of the heterotrimeric protein G12 ("$G\alpha_{12}$ mutant"). By "GTPase-deficient" is meant that the molecule lacks intrinsic GTPase activity. Hence, the $G\alpha_{12}$ mutant is constitutively active. By "constitutively activate" is meant that the activity of the protein is present essentially continually in the cell and is not subject to quantitative regulation. The $G\alpha_{12}$ mutant has the property of inducing the production of arachidonic acid. Arachidonic acid is a substrate of COX-2. COX-2 converts arachidonic acid into prostaglandins. The latter stimulate cell proliferation. The $G\alpha_{12}$ mutant also has the property of inducing the transcription of COX-2, which leads to the constitutive expression of COX-2 in cells engineered to express the mutant.

Contact of indicator cells with an inhibitor of COX-2 has the effect of inhibiting production of prostaglandins, and thus cell proliferation. At the same time, the prostaglandin precursor arachidonic acid will accumulate in the cell or culture medium due to the blockage of COX-2-mediated conversion of arachidonic acid to prostaglandin.

One such $G\alpha_{12}$ mutant is the protein $G\alpha_{12}Q229L$ ("$G\alpha_{12}QL$") described by Xu *et al.*, *supra*. The starting material for the $G\alpha_{12}QL$ (also referred to herein as "Q229L") mutant is the full-length murine wild-type $G\alpha_{12}$ cDNA disclosed by Strathman and Simon, *Proc. Natl. Acad. Sci.*

(USA) 1991; 88:5582-5582 or GenBank accession record M63659. The mutant is generated by the method described by Xu *et al.*, *supra*, utilizing site-directed mutagenesis of double-stranded DNA by overlap extension using PCR methodology. The mutagenic oligonucleotides have the following sequences, wherein the modified nucleotides are underlined: 5'-TGGGCGGCCTGAGGTCAC-3' (SEQ ID NO:1) and 5'-GTGACCTCAGGCCGCCCA-3' (SEQ ID NO:2).

Cells engineered to express $G\alpha_{12}QL$ are driven to proliferation through an autocrine loop shown in Fig. 1. COX-2 inhibitors can arrest cell proliferation by disrupting this autocrine loop. Such an inhibitory effect can be easily quantified by monitoring the $G\alpha_{12}QL$-stimulated synthesis of DNA. A putative COX-2 inhibitor can thus be easily and rapidly screened for COX-2 inhibitory activity.

Prior to the present invention, it was not known that the Q229L mutation induces COX-2 transcription in cells. It has been unexpectedly found that $G\alpha_{12}QL$-transformed cells constitutively express COX-2, thereby permitting their use as reagents for screening putative COX-2 inhibitors.

According to the invention, a host cell line capable of growth in culture, preferably a fibroblast line (e.g. NIH 3T3), is transfected with a construct which will drive $G\alpha_{12}QL$ DNA expression in the host cell line. The construct contains $G\alpha_{12}QL$ DNA and appropriate control elements. The construct may contain a non-inducible promoter, or an inducible promoter, e.g., an isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible promoter. Methods for establishing cell lines that expresses $G\alpha_{12}QL$ under control of an IPTG promoter are described by Vara Prasad *et al.*, *J. Biol. Chem.* 1995, 270:18655-18659, the entire disclosure of which is incorporated herein by reference.

Cells of the transfected cell line which produce arachidonic acid and COX-2 are then used as indicator cells for testing the COX-2 inhibitory activity of test substances. The test substance may comprise a pure chemical compound or mixture of chemical compounds. Typically, the test

substance will be an essentially pure chemical compound which may be contained in a suitable carrier for administration to the indicator cell culture. An appropriate concentration of test substance is introduced into the cell culture. An appropriate concentration can be determined by trial and error, without undue experimentation. The concentration of test substance may range, for example, from about 0.1 to about 100 μM, more typically from about 1 to about 20 μM, most typically from about 1 to about 10 μM.

According to one embodiment of the invention, the level of proliferation of the indicator cells with and without the test substance present in the cell culture is then determined. The test substance is contacted with the indicator cells under conditions which favor the proliferation of those cells. Methods for culturing cells under conditions of proliferation are well-known to those skilled in the art. Typically, conditions favoring cell proliferation in culture comprise culturing the cells in the presence of a growth medium, e.g., a growth medium comprising a serum such as fetal bovine serum (FBS). Assays for determining the level of proliferation of cells in culture are well-known to those skilled in the art. In addition to determining actual cell number, several indirect methods for assessing cell proliferation are known to those skilled in the art.

According to one preferred embodiment of a proliferation assay, proliferation is assessed by monitoring DNA synthesis. Proliferating cells actively synthesize DNA to support mitosis. DNA synthesis may be conveniently monitored through the cell's uptake of detectably labeled DNA precursors. Such labeled precursors include, for example, chemically-labeled and radiolabeled precursors. One chemically labeled precursor is bromodeoxyuridine. Bromodeoxyuridine incorporation can be detected by bromodeoxyuridine-antibodies in an enzyme-linked immunosorbent assay using fixed microcultures (Muir *et al.*, *Analytical Biochemistry* 1990; 185:377-382). The preferred radiolabeled precursor is tritium-labeled thymidine (^{3}H-thymidine). Other chemically labeled and radiolabeled DNA precursors suitable for use in cell proliferation assays are known to those

skilled in the art. The capacity of the test substance to inhibit cell proliferation is a measure of the potency of the substance as an inhibitor of COX-2 activity.

According to another embodiment of the invention, the level of one or more prostaglandins produced by the indicator cells, with and without the test substance present in the cell culture, be can determined. Such prostaglandins may include for example PGE_2, $PGF_{2\alpha}$, PGD_2, their stabilized chemical derivatives and the like. In samples were the chemical environment promotes the rapid turnover of prostaglandins such prostaglandins may be converted to stable derivatives prior to detection. Such stabilized derivatives include for example bicyclo prostaglandin E2, 8-isoprostane, and prostaglandin D2 methoxime which correspond to PGE_2, $PGF_{2\alpha}$, and PGD_2. A decrease in production of prostaglandins by the indicator cells in the presence of the test substances indicates that the test substance has COX-2 inhibitory activity. Assays for determining the level of prostaglandins produced by cells in culture are well-known to those skilled in the art. Such methods include, for example, chromatography-based techniques, receptor based assays or immunoassays for the prostaglandin(s) of interest. Receptor based prostaglandins assays may be based on the methods of Hanasaki *et al.* or Balapure *et al.* for example. Hanasaki *et al.*, J. Biol. Chem. 1990;265:4871-4875 and Balapure *et al.*, Biochem. Pharmacol. 1989;38:2375-2381. Prostaglandin production may be measured by the various methods in the media surrounding cells, in the cells themselves, or in a component of the cells. Components of the cells include for example organelles, membranes, proteins, cell lysates, cell pellets, cell supernatants and the like.

For example, prostaglandin production in radiolabeled cells may be assayed by a thin layer chromatography (TLC) as follows. Radiolabeled prostaglandins are extracted from cells, their components, or the surrounding media with an appropriate solvent such as ethyl acetate. The solvent-extracted radiolabeled prostaglandins are then spotted on a TLC

plate and developed under suitable conditions in an appropriate mobile phase. Radiolabeled prostaglandins may be detected by any suitable means such as phosporimaging, fluorography, scintillation counting, autoradiography and the like. Alternatives to TLC chromatography include any chromatography technique capable of discerning the relative amounts of prostaglandins produced by unlabeled or radiolabeled cells.

According to one preferred embodiment of the invention, prostaglandin production by the indicator cells is measured by a competitive immunoassay. In such an assay, a primary antibody specific to one or more prostaglandins is incubated with a sample to be analyzed followed by incubation of the sample with an immobilized secondary antibody specific to the primary antibody. A labeled prostaglandin is then incubated with the sample, and the labeled prostaglandin is allowed to compete with non-conjugated prostaglandins in the sample for prostaglandin binding sites on the primary antibody. The competing labeled prostaglandin may be labeled by any suitable means including radiolabeling, conjugation with a peroxidase or other easily detected polypeptide, chromophore labeling, or fluorophore labeling. Techniques for labeling prostaglandins are well known to those skilled in the art. The level of competing, labeled prostaglandin bound after washing is inversely related to the amount of prostaglandin present in the sample analyzed. (Shaw and Ramwell, *Methods of Biochem. Analysis* 1969; 17:325-371; Green *et al.*, *Advances in Prostaglandin and Thromboxane Res.* 1978; 5:15-38; Powell, *Prostaglandins* 1980; 20:947-957; Kelly *et al.*, *Prostaglandins, Leukotrienes and Essential Fatty Acids* 1989; 37:187-191; Granstrom and Kindhal, Advances in Prostaglandin and Thromboxane Res. 1980; 5:119-210; Morris *et al.*, *Prostaglandins* 1981; 21:771-778; Granstrom and Samuelsson, *Advances in Prostaglandin and Thromboxane Res.* 1978; 5:1-13). A competitive prostaglandin assay permits rapid, high throughput screening for COX-2 inhibitors using the indicator cells of the invention.

As an alternative to measuring prostaglandin production by the indicator cells, the level of arachidonic acid provided by the indicator cells is determined, with and without the test substance present in the cell culture. By a "level" of arachidonic acid which is "provided" by the indicator cells, is meant the relative amount or concentration of arachidonic acid in the cells or component thereof, or in the culture medium. Arachidonic acid is readily converted to prostaglandins in the presence of functional COX-2. An increase in the level of arachidonic acid by the indicator cells in the presence of the test substance indicates that the test substance has COX-2 inhibitory activity. Assays for determining the level of arachidonic acid produced by cells in culture are well-known to those skilled in the art. Arachidonic acid production may be measured in the media surrounding cells, in the cells themselves or in components of the cells. According to one preferred embodiment of the invention, arachidonic acid release is measured by labeling indicator cells with tritiated arachidonic acid ([^{3}H]-arachidonic acid), washing cells to remove free radiolabeled arachidonic acid, treating the cells with the test substances and after a time measuring by scintillation counting the radiolabeled arachidonic acid present in the media surrounding the cells. (Xu *et al.*, *supra*). In the absence of functioning COX-2 the radiolabelled arachadonic acid taken up by the cells is not converted to prostaglandin, but rather is released to the surrounding media.

The inhibitory compounds identified using the invention may be administered to individuals (animals, most particularly mammals including humans) afflicted with any disorder characterized by undesirable prostaglandin production resulting from cyclooxygenase activity, particularly COX-2 activity ("cyclooxygenase-mediated disorder"). In particular, COX-2 inhibitory compounds of the type identified using the invention are believed useful in treating inflamation and inflamation-related disorders, by administering to a subject having or susceptible to such inflamation or inflamation-related disorder and effective amount of an inhibitory

compound. Inflamation is associated with a variety of disease conditions. For a list of such disease conditions treatable by cyclooxygenase inhibitors, and COX-2 inhibitors in particular, see U.S. Patents 5,604,253 and 5,908,852, the entire disclosures of which are incorporated herein by reference. Such conditions include, for example, arthritis, including but not limited to rheumatoid arthritis, spondyloarthropathies, gouty arthritis, osteoarthritis, systemic lupus erythematosus and juvenile arthritis. Such conditions further include rheumatic fever, symptoms associated with influenza or other viral infections, common cold, low back and neck pain, dysmenorrhea, headache, toothache, sprains and strains, myositis, neuralgia, synovitis, gout and ankylosing spondylitis, bursitis, and following surgical and dental procedures. COX-2 inhibitory compounds are also believed useful as analgesics for treating or alleviating all forms of pain. The compounds are believed useful in the treatment of other disorders including asthma, bronchitis, tendinitis, bursitis; skin related conditions such as psoriasis, eczema, burns and dermatitis; gastrointestinal conditions such as inflammatory bowel disease, Crohn's disease, gastritis, irritable bowel syndrome and ulcerative colitis and for the prevention of colorectal cancer; the treatment of inflamation in such diseases as vascular diseases, migraine headaches, periarteritis nodosa, thyroiditis, aplastic anemia, Hodgkin's disease, sclerodoma, type I diabetes, myasthenia gravis, sarcoidosis, nephrotic syndrome, Behcet's syndrome, polymyositis, gingivitis, hypersensitivity, conjunctivitis, swelling occurring after injury, myocardial ischemia, and the like. The COX-2 inhibitory compounds identified using the invention are also believed useful as antipyretics for the treatment of fever.

In addition, COX-2 inhibitors of the type identified using the invention may inhibit cellular neoplastic transformations and metastatic tumor growth and hence can be used in the treatment of cancer. The term "neoplasia" includes neoplasias that produce prostaglandins or express a cyclooxygenase, including both benign and cancerous tumors, growths and

polyps. Neoplasias believed treatable with cyclooxygenase inhibitors are discussed in U. S. Pat. 5,972,986, the entire disclosure of which is incorporated herein by reference. The COX-2 inhibitors identified using the invention may be used to inhibit the growth of an established neoplasm, i.e., to induce regression, or to prevent or delay the onset of the neoplasm.

According to U.S. Pat. 5,972,986, neoplasias that produce prostaglandins, and which are therefore believed treatable with compounds of the type identified using the invention, include brain cancer, bone cancer, epithelial cell-derived neoplasia (epithelial carcinoma) such as basal cell carcinoma, adenocarcinoma, gastrointestinal cancer such as lip cancer, mouth cancer, esophageal cancer, small bowel cancer and stomach cancer, colon cancer, liver cancer, bladder cancer, pancreas cancer, ovary cancer, cervical cancer, lung cancer, breast cancer and skin cancer, such as squamous cell and basal cell cancers, prostate cancer, renal cell carcinoma, and other known cancers that effect epithelial cells throughout the body.

The COX-2 inhibitory compounds identified by using the invention may also be useful in the treatment of angiogenesis-mediated disorders. Angiogenesis-mediated disorders which may be treatable with cyclooxygenase inhibitors are discussed in U. S. Pat. 6,025,353, the entire disclosure of which is incorporated herein by reference. According to U. S. Pat. 6,025,353, such disorders include, for example, metastasis, corneal graft rejection, ocular neovascularization, retinal neovascularization, diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, gastric ulcer, infantile hemaginomas, angiofibroma of the nasopharynx, avascular necrosis of bone, and endometriosis.

The practice of the invention is illustrated by the following non-limiting examples.

Example 1

Establishing $G\alpha_{12}QL$ Cell Line

A. Transfection of NIH3T3 Cells with $G\alpha_{12}QL$ Vector

(i) Preparation of $G\alpha_{12}QL$-NIH3T3

The vector $G\alpha_{12}QL$-pcDNA3 was prepared by ligating the EcoRI-XbaI (1.8 kb) fragment from pcDNA1-$\alpha_{12}Q229L$ into the EcoRI-XbaI site of the pcDNA3 vector (Invitrogen, Carlsbad, CA). Actively proliferating NIH 3T3 cells (1.5 x 10^6 cells/ 100 mm dish) grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS were transfected with 100 ng of the vector by the calcium precipitation method. The procedures for transfection and transformation of NIH 3T3 cells have been previously described (Vara Prasad *et al.*, *Oncogene* 1994, 9:2425-2429, and Shore & Reddy, *Oncogene* 1989, 4:1411-1423, the entire disclosures of which are incorporated herein by reference.) Individual transformed foci were isolated and expanded for further analysis by Northern and Western analyses for $G\alpha_{12}QL$ expression as well as anchorage-independent growth in soft-agar according to previously published procedures (Vara Prasad *et al.*, 1994, *supra*).

(ii) Preparation of IPTG-inducible $G\alpha_{12}QL$-NIH3T3

An NIH3T3 cell line that expresses $G\alpha_{12}QL$ under an IPTG-inducible promoter ($G\alpha_{12}QL$-pOPRSVI-NIH3T3) was established as previously described (Vera Prasad *et al.*, 1995, *supra*), as follows. Blunt-ended 1.8 kb HindIII-XbaI fragment of $\alpha_{12}Q229L$ excised from the $G\alpha_{12}QL$-pcDNA3 vector was ligated into the blunted NotI site of the pOPRSVI plasmid following published procedures (Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). NIH3T3 cell lines expressing inducible $\alpha_{12}Q229L$ were established using the LacSwitch expression vectors (Stratagene). Briefly, NIH3T3 cell were cotransfected with p3'SS plasmid vector expressing the Lac repressor and pOPRSVI-$\alpha_{12}Q229L$ vector by electroporation, as described by Vera Prasad *et al.*, 1995.

B. Preparation of COX-2 DNA

Murine COX-2 DNA was cloned using RT-PCR methods from the RNA prepared from NIH 3T3 cells pretreated (6 hrs) with 100 nM PMA using the following primers: 5'CTCTGCGATGCTCTTCCGAG-3' (SEQ ID NO:3) and 5'-GACTTTTACAGCTCAGTTGAACG-3' (SEQ ID NO:4). The amplified 1827 bp PCR-product was sequenced and the comparison with the published sequences (Kujubu *et al.*, *J. Biol. Chem.* 1991, 266, 12866-12872) confirmed the identity of COX-2 cDNA. The COX-2 PCR-product was purified and cloned into pT7Blue TA vector (Novagen, Madison, WI). The clone was used as a source of probes for Northern blot analysis.

C. Confirmation of COX-2 Expression in IPTG-inducible $G\alpha_{12}$QL-NIH3T3 Cells

1. Northern Blotting

Expression of $G\alpha_{12}$QL was induced in the IPTG-inducible$G\alpha_{12}$-QL-NIH3T3 cells and control cells by the addition of 1 mM IPTG. At 0, 0.5, 1, 3, and 6 hours after the addition of IPTG, total RNA was prepared from the cells following the published procedures of Dhanasekaran *et al.*, *J. Biol. Chem.* 1993, 269:11802-11806, the entire disclosure of which is incorporated herein by reference. Twenty µg of the RNA (for each time point) was resolved in a denaturing 1 % agarose and 2.2 M formaldehyde gel. The RNA was blotted onto a zeta probe-GT membrane (Bio-Rad, CA) and cross-linked to the membrane by UV light. The Hind III-Xba I fragment (1.2 kbp) of $G\alpha_{12}$QL DNA excised from $G\alpha_{12}$QL and the Xba I-Bam HI fragment (1.8 kbp) of COX-2 from the COX-2pT7Blue vector were used as probes in Northern blot analyses. The RNA was also probed for GAPDH expression.

The results of the Northern blot analysis are shown in Figs. 2A ($G\alpha_{12}$QL-3NIH3T3) and 2B (control cells). The control cells were NIH3T3 cells transfected with "empty" pOPRSVI vector lacking the $G\alpha_{12}$QL insert.

COX-2 transcription was induced only in $G\alpha_{12}$QL-NIH3T3 cells.

2. Western Blotting

The constitutive expression of COX-2 protein was demonstrated by Western blot analysis. Cell lysates for Western blot analysis were prepared from control NIH3T3, NIH3T3 expressing wild type $G\alpha_{12}$ ($G\alpha_{12}$WT-NIH3T3) and $G\alpha_{12}$QL-NIH3T3 cells. The cells grown in 100 mm dishes, washed twice in phosphate-buffered saline (PBS) and lysed with 0.5 ml of RIPA buffer (10 mM $NaPO_4$ pH 7.0, 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 1% NP40, 0.1% SDS, 50 mM NaF, 200 mM Na_3VO_4, 0.1% β-mercaptoethanol, 1 mM phenylmethanesulfonyl-fluoride (PMSF), 4 µg/ml aprotinin, and 2 µg/ml leupeptin). The soluble proteins were cleared by centrifugation at 15, 000 g for 10 minutes at 4°C. The lysate (50 µg) was resolved by SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) in 10 mM 3-cyclohexylamino-1-propane sulfonic acid (CAPS) buffer containing 10% methanol using a Mini-Protean apparatus (Bio-Rad, CA). The resolved lysates were probed with specific antibodies raised against the C-terminus of COX-2 (Oxford Biomedical Research, MI).

The results are shown in Fig. 3. COX-2 is constitutively expressed in $G\alpha_{12}$QL-NIH3T3 cells (lanes 3 and 6) but not in control NIH3T3 cells (lanes 1 and 4) or $G\alpha_{12}$WT-NIH3T3 cells (lanes 2 and 5).

Example 2

Inhibition of $G\alpha_{12}$QL-NIH3T3 Cell Proliferation by COX-2 Inhibitor

Inhibition of $G\alpha_{12}$QL-NIH3T3 cell proliferation by a COX-2 inhibitor was determined by monitoring DNA synthesis. [^{3}H]-Thymidine incorporation was used as an index of DNA-synthesis. Control cells (expressing pcDNA3 vector) and $G\alpha_{12}$QL-transformed NIH 3T3 cells were plated at 5 x 10^4 cells per well in a 24-well plate and grown for 24 hours at 37°C in DMEM containing 5 % FBS. The cells were washed

and made quiescent by placing them in DMEM (+0.2 % BSA) without serum for an additional 24 hours. Cell growth was reinitiated by the addition of 10 % FBS with or without an experimental COX-2 inhibitor (10 μM). Twelve hours later 1 μCi of thymidine (20 Ci/nmol) was added to each well and incubated for an additional 4 hours. The cells were washed in PBS and solubilized in 0.1% SDS. DNA was precipitated with 10 % chilled TCA and the precipitate was collected on GFC filters. The counts in the precipitate were determined by scintillation counting. The results are shown in Fig. 4. The values represent the mean ± the standard error for triplicate determinations.

The addition of 10 μM COX-2 inhibitor did not have any effect on control cells. In contrast, the COX-2 inhibitor specifically inhibited DNA synthesis in $G\alpha_{12}$QL-NIH3T3 cells as shown in Fig. 4. These results indicated that the COX-2-inhibitor did not affect normal cells but arrested cell growth in transformed cells dependent on the COX-2 pathway for cell growth and survival. Since $G\alpha_{12}$QL-NIH3T3 cells exhibit such a phenotype, the bioassay system based on $G\alpha_{12}$QL-NIH3T3 cells may be effectively used to screen putative COX-2 inhibitors.

Example 3

Determination of Prostaglandin Levels as an Assay for COX-2 Activity

Activation of prostaglandin release by NIH3T3 cells in response to $G\alpha_{12}$QL expression was determined by monitoring prostaglandin production. Prostaglandin PGE_2 was used as an index of COX-2 mediated prostaglandin production. Control NIH3T3 cells stably transfected with pcDNA3 vector and $G\alpha_{12}$QL-transformed NIH3T3 cells were plated at 3 x 10^6 cells per 100 mm dish and grown overnight at 37°C in DMEM containing 5 % FBS. The cells were washed and made quiescent by placing them in DMEM (+0.2 % BSA) without serum for an additional 24

hours. Cell growth was reinitiated by the addition of 10% FBS. Six hours later the cells were pelleted by centrifugation. The cell pellet was lysed and total cellular PGE_2 production was measured using a competitive enzyme immunoassay (Prostaglandin E_2 EIA (enzymeimmunoassay) Kit, Cayman Chemical Co., Ann Arbor, MI, USA) as per the manufacturer's protocol. The results are shown in Fig. 5. The values represent the mean ± the standard error for triplicate determinations.

Stable expression of $G\alpha_{12}QL$ dramatically activated prostaglandin PGE_2 production in the indicator cells in contrast to control NIH3T3 cells stably transfected with pcDNA3 as shown in Fig. 5. Activation or inhibition of COX-2 activity in $G\alpha_{12}QL$ transfected indicator cells can be assessed by determining the levels of prostaglandins produced by such indicator cells. The assay may be used to screen candidate compounds for inhibition of COX-2 activity.

Example 4

Determination of Arachidonic Acid Levels as an Assay for COX-2 Activity

Arachidonic acid release levels can be used as an index of COX-2 activity. Control NIH3T3 cells stably transfected with pcDNA3 vector and $G\alpha_{12}QL$-transformed NIH3T3 cells were plated at 2 x 10^4 cells per well in a 24-well plate and grown for 24 hours at 37°C in DMEM containing 5 % FBS. Cells were labeled and made quiescent by incubation for 24 hrs with 0.5 ml serum free DMEM containing 10 mM HEPES (ph 7.4), 0.2 % BSA and 0.5 μCi [^{3}H]-arachidonic acid per well. Cells were then washed three times with PBS to remove free [^{3}H]-arachidonic acid. Cell growth was reinitiated by the addition of FBS to a final concentration of 5 %. [^{3}H]-Arachidonic acid release into the surrounding media was measured at 0, 2, 5, 10, and 20 min after serum stimulation by scintillation counting as per the method of Xu *et al., supra*. Prior to scintillation counting cell debris in the media was removed by centrifugation at 2000 g. [^{3}H]-arachidonic acid

release by non-serum stimulated cells into the surrounding media was measured at 0, 2, 5, 10, and 20 min by scintillation counting. The results are shown in Fig. 6A. The values represent the mean ± the standard error for triplicate determinations.

Serum stimulation of $G\alpha_{12}QL$ transfected cells dramatically increased arachidonic acid release in contrast to non-serum stimulated indicator cells as shown in Fig. 6A. Arachidonic acid release by control NIH3T3 cells stably transfected with pcDNA3 was unaffected by serum stimulation as shown in Fig. 6A. Activation or inhibition of COX-2 activity in $G\alpha_{12}QL$ transfected indicator cells can be assessed by determining the levels of arachidonic acid produced by such indicator cells. Inhibition of COX-2 will presumably increase arachidonic acid levels as such inhibition will prevent conversion of arachidonic acid into the various prostaglandins.

Arachidonic acid release levels were also used as an index of COX-2 activity in NIH3T3 cells stably transfected with pcDNA3, *ras*, $G\alpha_{12}QL$, and $G\alpha_{13}QL$ expression vectors as shown in Fig. 6B. Cells were plated, labeled with [^{3}H]-arachidonic acid, and [^{3}H]-arachidonic acid release was measured using the methodologies described above. [^{3}H]-arachidonic acid release was measured by scintillation counting 10 min after reinitiation of cell growth by addition of 5% FBS. The results are shown in Fig. 6B. The values represent the mean ± the standard error for quadruplicate determinations.

As Fig. 6B shows, serum stimulation of $G\alpha_{12}QL$ transfected indicator cells dramatically increased arachidonic acid release relative to cells stably transfected with pcDNA3, *ras*, or $G\alpha_{13}QL$ expression vectors as shown in Fig. 6A. These results demonstrate the apparent specificity with which $G\alpha_{12}QL$ expression stimulates the COX-2 pathway. Activation or inhibition of COX-2 activity in $G\alpha_{12}QL$ transfected indicator cells can be assessed by determining the levels of arachidonic acid produced by such indicator cells. The assay may be used to screen candidate compounds for inhibition of COX-2 activity.

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference. All sequence records identified by GenBank accession numbers are incorporated herein by reference.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indication the scope of the invention.

What is claimed is:

1. A method for screening a test substance for COX-2 inhibitory activity comprising:

(a) contacting the test substance with indicator cells which constitutively express endogenous COX-2 or inducibly express endogenous COX-2; and

(b) determining the level of:

(i) proliferation of the indicator cells in the presence and absence of the test substance, a decreased level of proliferation of the indicator cells in the presence of the test substance indicating that the test substance has COX-2 inhibitory activity; or

(ii) one or more prostaglandins produced by the indicator cells in the presence and absence of the test substance, a decreased prostaglandin level in the presence of the test substance indicating that the test substance has COX-2 inhibitory activity.

(iii) arachidonic acid produced by the indicator cells in the presence and absence of the test substance, an increased arachidonic acid level in the presence of the test substance indicating that the test substance has COX-2 inhibitory activity.

2. A method for screening a test substance for COX-2 inhibitory activity comprising:

(a) contacting the test substance with indicator cells which express a GTPase-deficient mutant form of the α-subunit of protein G12, which mutant α-subunit has the capacity to induce the production of arachidonic acid and COX-2 in the indicator cells; and

(b) determining the level of proliferation of the indicator cells in the presence and absence of the test substance, a decreased level of proliferation of the indicator cells in the presence of the test substance indicating that the test substance has COX-2 inhibitory activity.

3. A method according to claim 2 wherein the G12 protein α-subunit mutant comprises the Q229L mutation.

4. A method according to claim 3 wherein the level of indicator cell proliferation is determined by an assay for DNA synthesis by the indicator cells.

5. A method according to claim 4 wherein the DNA synthesis assay comprises assaying tritium-labeled thymidine uptake by the indicator cells.

6. A method for screening a test substance for COX-2 inhibitory activity comprising:

(a) contacting the test substance with indicator cells which express a GTPase-deficient mutant form of the α-subunit of protein G12, which mutant α-subunit has the capacity to induce the production of arachidonic acid and COX-2 in the indicator cells; and

(b) determining the level of one or more prostaglandins produced by the indicator cells in the presence and absence of the test substance, a decrease in production of said prostaglandin by the indicator cells in the presence of the test substance indicating that the test substance has COX-2 inhibitory activity.

7. A method according to claim 6 wherein the G12 protein α-subunit mutant comprises the Q229L mutation.

8. A method according to claim 7 wherein the prostaglandin level is assayed in the media surrounding the indicator cells, in the indicator cells or in a component of the indicator cells.

9. A method according to claim 8 wherein the prostaglandin level

level is assayed by a prostaglandin immunoassay.

10. A method according to claim 9 wherein the prostaglandin immunoassay comprises a competitive immunoassay.

11. A method for screening a test substance for COX-2 inhibitory activity comprising:

(a) contacting the test substance with indicator cells which express a GTPase-deficient mutant form of the α-subunit of protein G12, which mutant α-subunit has the capacity to induce the production of arachidonic acid and COX-2 in the indicator cells; and

(b) determining the level of arachidonic acid provided by the indicator cells in the presence and absence of the test substance, an increase in the level of arachidonic acid provided by the indicator cells in the presence of the test substance indicating that the test substance has COX-2 inhibitory activity.

12. A method according to claim 11 wherein the G12 protein α-subunit mutant comprises the Q229L mutation.

13. A method according to claim 12 wherein the arachidonic acid level is assayed in the media surrounding the indicator cells, in the indicator cells or in a component of the indicator cells.

14. A method according to claim 13 wherein the arachidonic acid assay comprises assaying tritium labeled arachidonic acid release from said indicator cells.

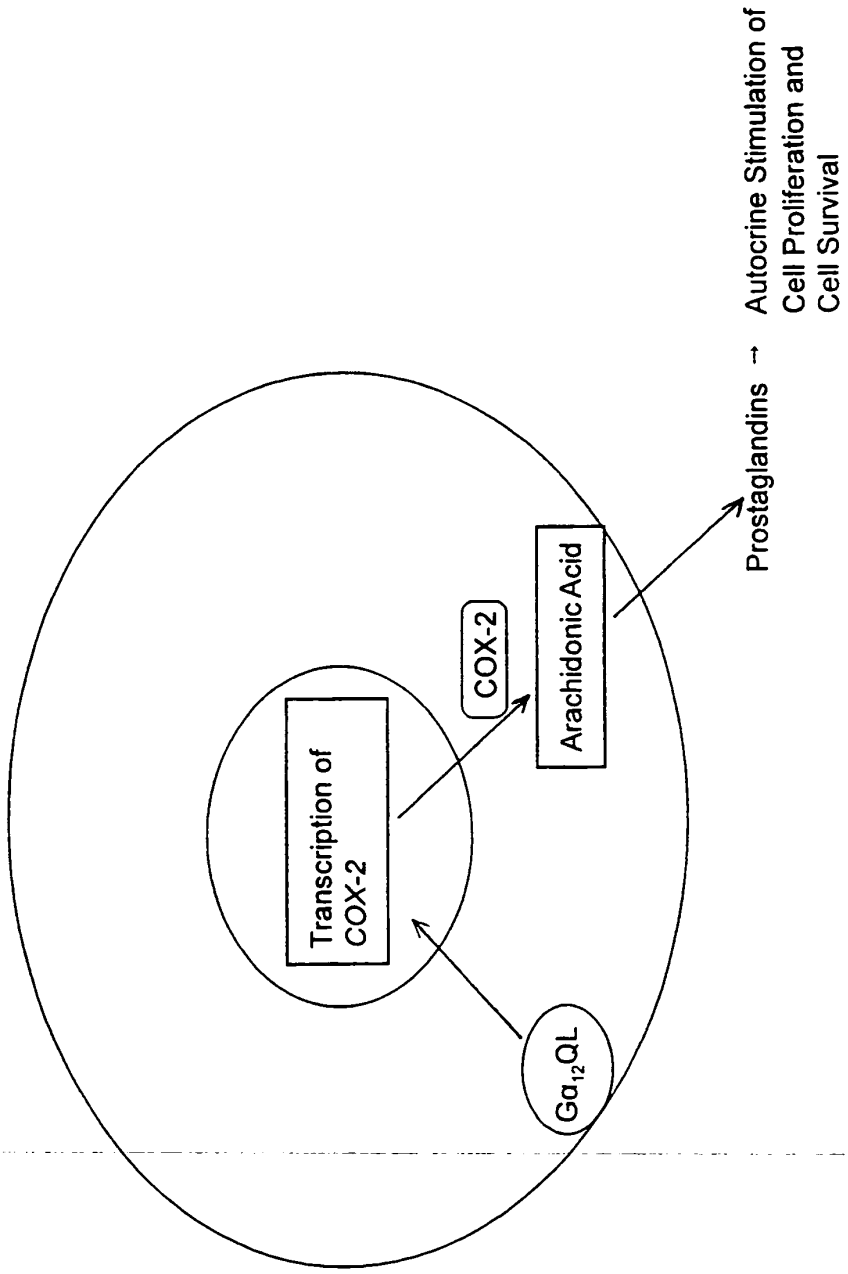

Autocrine Stimulation of
Cell Proliferation and
Cell Survival
Prostaglandins
Arachidonic Acid
COX-2
Transcription of
COX-2
$G\alpha_{12}QL$

FIG. 1

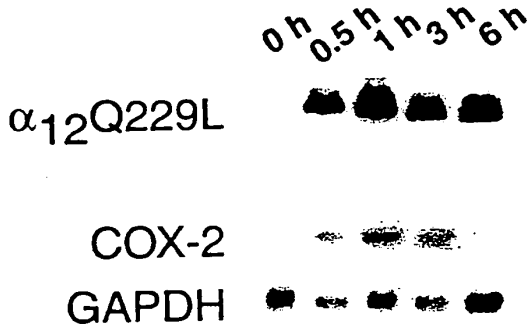
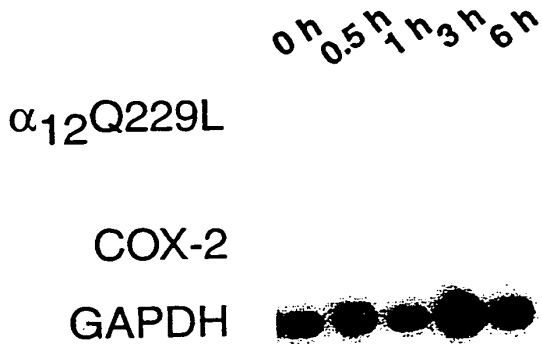

0h 0.5h 1h 3h 6h
α12Q229L
COX-2
GAPDH

FIG. 2A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α12Q229L
COX-2
GAPDH

FIG. 2B

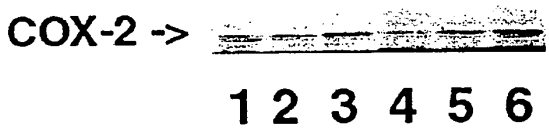

FIG. 3

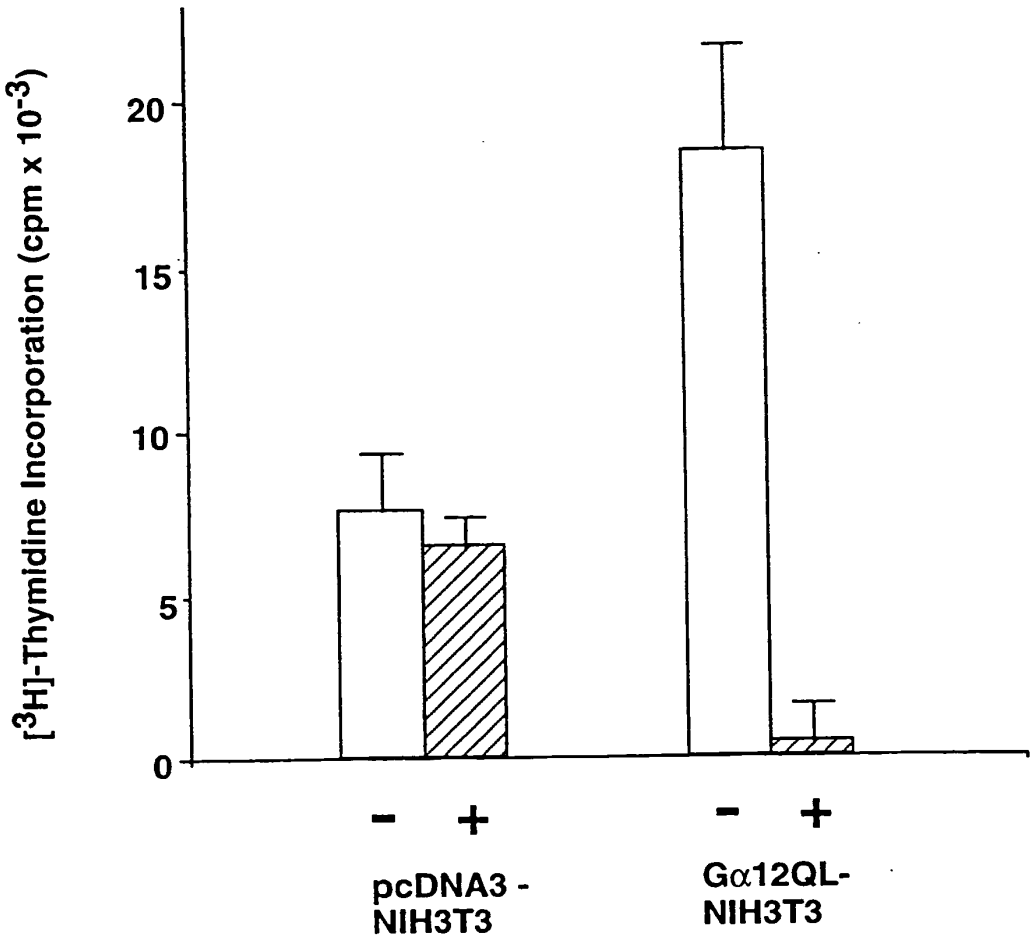
[3H]-Thymidine Incorporation (cpm x 10-3)
20
15
10
5
0
- +
- +
pcDNA3 - NIH3T3
Gα12QL- NIH3T3

FIG. 4

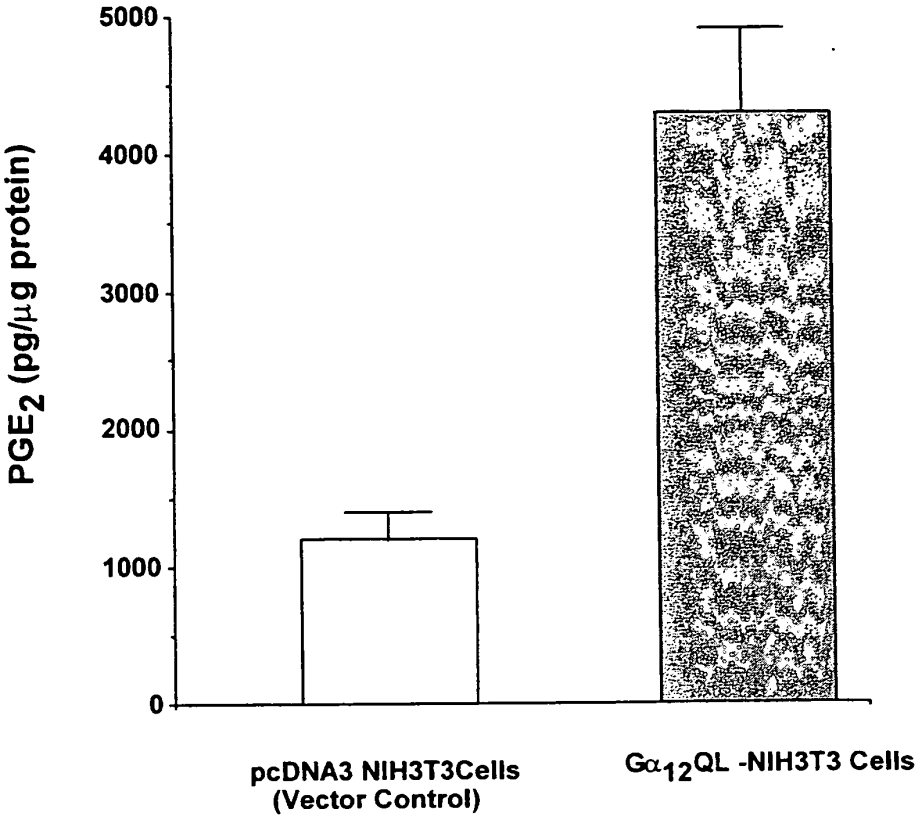
5000
4000
3000
2000
1000
0
PGE2 (pg/µg protein)
pcDNA3 NIH3T3Cells
(Vector Control)
Gα12QL -NIH3T3 Cells

FIG. 5

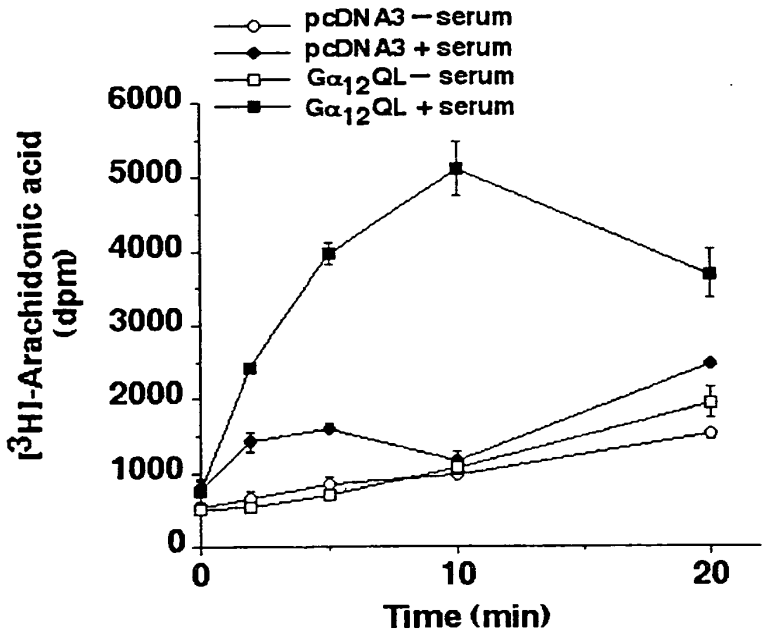
pcDNA3 – serum
pcDNA3 + serum
Gα12QL – serum
Gα12QL + serum
[3H]-Arachidonic acid (dpm)
6000
5000
4000
3000
2000
1000
0
0
10
20
Time (min)

FIG. 6A

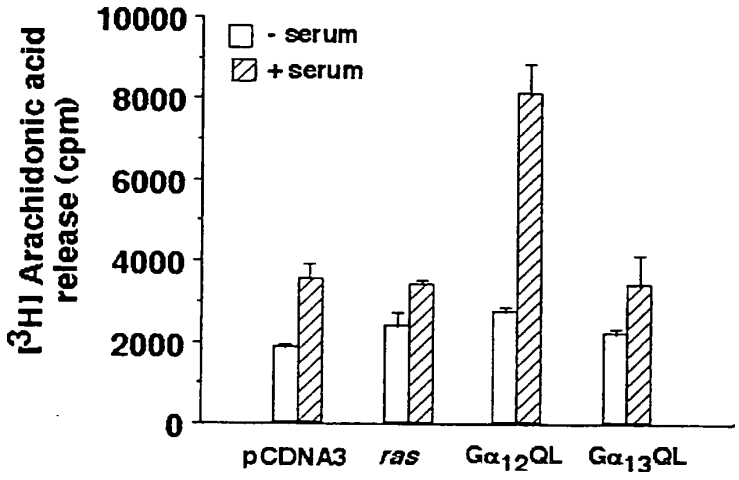
[3H] Arachidonic acid release (cpm)
10000
8000
6000
4000
2000
0
- serum
+ serum
pCDNA3
ras
Gα12QL
Gα13QL

FIG. 6B

SEQUENCE LISTING

<110> Temple University - Of The Commonwealth System of Higher Education
Reddy, E. Premkumar
Reddy, M.D. Ramana
Dhanasekaran, N.

<120> Cell-Based Assay For Screening Cox-2 Inhibitors

<130> 6056-268 PC

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<151> 1999-06-16

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<220>
<223> Primer for cloning murine COX-2 DNA

<400> 4
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16486

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C12Q 1/26, 1/28; C12N 9/00, 9/02
US CL :435/7.2, 7.21, 25, 28, 183, 189
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.2, 7.21, 25, 28, 183, 189

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, CA, CAPLUS, MEDLINE, EMBASE, SCISEARCH, CANCERLIT, USPTO-WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAUFER et al. Development of an in-vitro test system for the evaluation of cyclooxygenase-2 inhibitors. Inflamm. Res. 01 March 1999, Vol. 48, pages 133-138, see entire document.	1-15
Y	BRIDEAU et al. A human whole blood assay for clinical evaluation of biochemical efficacy of cyclooxygenase inhibitors. Inflamm. Res. 01 February 1996, Vol. 45, pages 68-74 see entire document, especially page 68, column 2; page 69, column 2.	1-15
Y	MCCORMACK, K. Roles of COX-1 and COX-2. J. Rheumatol. 01 November 1998, Vol. 25, pages 2279-2280, see entire document.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
03 OCTOBER 2000	17 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MANJUNATH RAO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/16486

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	XU, N. et al. A mutant α subunit of G_{12} potentiates the eicosanoid pathway and is highly oncogenic in NIH 3T3 cells. Proc. Natl. Acad. Sci. USA. 01 July 1993, Vol. 90, pages 6741-6745, see entire document.	1-15

Ann. Rev. Biochem. 1986. 55:69–102

ARACHIDONIC ACID METABOLISM[1]

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CONTENTS

[1]Investigations by the authors in areas discussed in this chapter have been supported in part b grants from the National Institutes of Health: (JT) AM34388; (BAJ) HL21874 and HL31922; (JL NIH Clinical Investigator Award HL01313; (ARM) AM30542 and (PN) 5-P50-HL1764(HL14397, and HL20787.

[2]Department of Medicine

The synthesis and function of arachidonic acid metabolites remains the focus of extensive investigation. This subject has previously been reviewed in the *Annual Review of Biochemistry* in 1975, 1978, and 1983. The current effort is organized into a brief consideration of the products and function of the major synthetic enzymes, namely cyclooxygenase, the endoperoxide-dependent enzymes, 12-lipoxygenase, 15-lipoxygenase, 5-lipoxygenase, leukotriene synthetic enzymes, and cytochrome p450. In addition, attention is directed at the role and function of arachidonic acid metabolism in certain models of pathophysiological conditions. Finally, recent advances in the study of essential fatty acid deficiency, especially as related to arachidonic acid metabolism, warrant consideration.

CYCLOOXYGENASE PRODUCTS

Prostaglandin Endoperoxide Synthase

Arachidonic acid (1, 2) and certain other polyunsaturated fatty acids (3–5) may be transformed into prostaglandins (PG) by the enzyme prostaglandin endoperoxide synthase (PES) (1, 2, 6). The cyclooxygenase activity of PES inserts two molecules of oxygen into arachidonate to yield a 15-hydroperoxy-9,11-endoperoxide with a substituted cyclopentane ring (PGG_2). A peroxidase activity of PES reduces PGG_2 to its 15-hydroxy analogue (PGH_2). The cyclooxygenase and peroxidase activities of PES reside in a single protein (2, 6, 7). PES is membrane-associated but has been detergent-solubilized and purified to homogeneity (7, 8). The subunit molecular weight of the purified enzyme is about 72,000 (7, 8). Each subunit requires one molecule of heme for maximal catalytic activity (8). As recently reviewed (6), the specific activity of the enzyme ranges between 2.4 and 23.4 μmoles/min/mg at 24°C, and the K_m for both arachidonate and 0_2 is about 5 μM. Immunocytochemical studies indicate that PES is contained in endoplasmic reticulum and nuclear membrane but not plasma membrane or mitochondrial membrane of cultured fibroblasts (9).

Cyclooxygenase-catalyzed fatty acid oxidation occurs slowly initially and later accelerates (10). Exogenous hydroperoxides eliminate this kinetic lag phase at concentrations (10^{-7} to 10^{-8} M) far below the K_m (10^{-5} M) of the peroxidase activity of PES (11). A continuous requirement for activator hydroperoxide is implied by termination of PES-catalyzed substrate oxidation upon addition of glutathione and glutathione peroxidase (10). Cyclooxygenase-catalyzed oxygen consumption declines to zero before complete consumption of fatty acid substrate, and a second burst of oxygen consumption occurs upon addition of fresh enzyme (10). Such self-deactivation of the cyclooxygenase appears to occur in intact cells as well as with purified enzyme preparations and may limit in vivo prostaglandin biosynthesis (12, 13).

The peroxidase activity of PES exhibits a K_m of about 20 μM for PGG_1,

catalyzes the reduction of other lipid hydroperoxides and H_2O_2, and also undergoes self-deactivation (14, 15). Various reducing cosubstrates increase the number of turnovers before deactivation, and these cosubstrates may be covalently modified during this process (16, 17). Cosubstrate oxidation by PES has been postulated to activate certain procarcinogenic substances (17, 18).

Nonsteroidal anti-inflammatory agents inhibit the cyclooxygenase but not the hydroperoxidase activity of PES (19). As recently reviewed (6), aspirin acetylates the enzyme resulting in irreversible inhibition; certain acetylenic fatty acids, such as eicosa-5,8,11,14-tetrynoic acid (ETYA), also inactivate the cyclooxygenase, possibly by acting as suicide substrates.

Metabolism of the Prostaglandin Endoperoxides

THROMBOXANES In blood platelets the principal metabolite of PGG_2 and PGH_2 is thromboxane A_2 (TxA_2). The oxetane ring of TxA_2 spontaneously hydrolyzes ($t_{1/2}$ = 30 sec) to the hemiacetal thromboxane B_2 (TxB_2) (2). TxA_2 but not TxB_2 contracts vascular smooth muscle and induces platelet aggregation and serotonin release at concentrations below 20 nM.

Platelet thromboxane synthase activity is associated with dense tubular membranes and catalyzes formation of 12-hydroxy-heptadeca-trienoic acid (HHT) as well as TxA_2 from PGH_2. Some HHT must also arise from the nonenzymatic breakdown of PGH_2. A 750-fold purification of the enzyme has been achieved by affinity chromatography with an immobilized inhibitor of thromboxane synthase (20). Similar enrichment for thromboxane synthase activity and for cytochrome p450 optical properties during the purification suggest that thromboxane synthase may be a cytochrome p450–type hemoprotein (20).

Inhibitors of thromboxane synthase include imidazole (21), certain substituted imidazoles (such as dazoxiben) (22), certain pyridine derivatives (such as OKY-1581) (23), prostaglandin analogues containing a pyridine rather than a cyclopentane ring (24), and PGH_2 analogues such as 15-deoxy-9,11-azo-PGH_2 (25). The compound 9,11-azo-PGH_2 mimics the action of thromboxane as does 9,11-methanoepoxy-PGH_2 (U46619) (26).

Thromboxane synthase inhibitors prevent TxA_2 formation but not aggregation by washed platelets in response to arachidonate (21). Coupled with the TxA_2-mimetic properties of stable endoperoxide analogues such as U44619, this has suggested that TxA_2 and PGH_2 may activate platelets by a common receptor (27). Certain analogues of PGH_2 (e.g. 15-deoxy-9,11-epoxyimino-PGH_2) and of TxA_2 (e.g. pinane-TxA_2) prevent PGH_2-induced platelet aggregation at concentrations that do not inhibit thromboxane synthase (26, 28). Such compounds may represent antagonists at a putative TxA_2/PGH_2 receptor (26–28).

Thromboxane B_2 infused intravenously into man is rapidly metabolized to a variety of compounds that are excreted into the urine, and the most abundant of these is 2,3-dinor-TxB_2 (29). No circulating plasma metabolite has been identified, and peripheral blood TxB_2 derives largely from platelet activation during blood collection (30).

PROSTACYCLIN Vascular endothelial cells convert PGH_2 to prostacyclin (PGI_2), an enol-ether which spontaneously hydrolyzes ($t_{1/2}$ = 10 min at pH 7.4, 24°C) to 6-keto-$PGF_{1\alpha}$ (31). These compounds were first identified from rat stomach (32), and PGI_2 was subsequently shown to be responsible for arachidonate-induced coronary vasodilation observed by Needleman and coworkers (33, 34) and for the antiaggregatory influence on platelets of medium from incubation of PGH_2 and aortic microsomes observed by Vane and coworkers (35). Vascular and nonvascular smooth muscle cells also synthesize PGI_2 (36, 37), although endothelial cells have a higher synthetic capacity due to a higher cyclooxygenase content than myocytes (37). Not all endothelial cells synthesize PGI_2 (38).

PGI_2 synthase has been purified to homogeneity by affinity chromatography using immobilized monoclonal antibody to PGI_2 synthase activity (39) or immobilized endoperoxide substrate analogue (40). Immunofluorescence studies indicate that the enzyme is located in the plasma membrane and nuclear membrane of a wide variety of smooth muscle cells (36). The purified enzyme exhibits a subunit molecular weight of about 50,000 and a specific activity of 1000–2000 μmole PGI_2/min/mg protein (39, 40). The K_m for PGH_2 is about 5 μM (41) and self-deactivation occurs during catalysis (39). The optical behavior of the purified enzyme suggests that it may be a cytochrome p450–type hemoprotein (42). PGI_2 synthase is inactivated by a variety of lipid hydroperoxides (41, 43), a process that is partially prevented by reducing compounds that are radical scavengers (43).

Thrombin stimulates PGI_2 synthesis by cultured endothelial cells (44), and ADP stimulates PGI_2 synthesis by isolated rabbit aorta (45). TxA_2-mimetic PGH_2 analogues stimulate PGI_2 synthesis by cultured vascular smooth muscle cells (46). Platelet-derived PGH_2 also appears to be utilized by endothelial cells for PGI_2 synthesis (47, 48), and this effect is magnified by thromboxane synthase inhibitors (49). Augmentation of vascular cell PGI_2 production by platelet activators and platelet release products may serve to limit the area of platelet deposition about a site of vascular injury. After endothelial cells have been stimulated to produce PGI_2, they become unresponsive to a second stimulus, apparently due to deactivation of the cyclooxygenase (12, 13). Vascular PGI_2 synthesis may therefore occur in phasic bursts which must be actively initiated rather than as a continuous process.

The vasodepressor substances histamine (50) and bradykinin (51) also stimulate PGI_2 synthesis by cultured endothelial cells, and this response coul mediate a component of the depressor effects of these compounds in vivo. PGI production by cultured endothelial cells declines as the cells reach confluenc and increases when the cells are seeded into fresh medium at lower density (52 Similar phenomena occur with cultured 3T3-L1 preadipocytes (53), and e> ogenous PGI_2 retards while indomethacin promotes the differentiation of thes cells into mature adipocytes (54). PGI_2 may therefore modulate the pr liferative and differentiation state of some cells.

PGI_2 elevates cAMP levels in platelets (55), vascular smooth muscle cell (56), and toad bladder epithelium (57), and this biochemical action appears t mediate the antiaggregatory effects of PGI_2 on platelets (55), the activation c myocyte cholesterol ester hydrolase (56), and increase in toad bladder sho circuit current (57). Activation of adenylate cyclase via a PGI_2 receptor ma represent a general mechanism for the effects of PGI_2 on responsive cell: Membranes from a cultured, PGI_2-responsive cell line have been reported t contain a specific protein receptor for PGI_2 with a maximum binding capacit of 350 fmol/mg membrane protein and a molecular weight of 82,000 (58)

PGI_2 and 6-keto-$PGF_{1\alpha}$ infused intravenously into man are rapidly metab lized to a variety of compounds, and the principal urinary metabolite is 2,3 dinor-6-keto-$PGF_{1\alpha}$ (59). Circulating levels of PGI_2 (measured as 6-ket $PGF_{1\alpha}$) in human plasma are less than 3 pg/ml, which is insufficient to exert an recognized biologic activity (60). Circulating metabolites of PGI_2 incluc 2,3-dinor-13,14-dihydro-6,15-diketo $PGF_{1\alpha}$ and its 20-carboxy analogue (61 The compound 6-keto-PGE_1 is produced from 6-keto-$PGF_{1\alpha}$ upon perfusio through rabbit liver (62), and this compound inhibits platelet aggregatio although less potently than PGI_2 (63).

PROSTAGLANDIN D_2 Isomerization of PGH_2 to PGD_2 can be catalyzed b serum albumin (K_m 6 μM, specific activity 0.87 mol PGH_2/min/mol albumin 37°C) (64). PGH to PGD isomerase activity has been purified to electrophoret homogeneity from the cytosol of rat brain (65) and rat spleen (66). The brai enzyme has a molecular weight of 80,000–85,000, a K_m for arachidonate of μM, and requires glutathione. The spleen enzyme does not require glutathior and has a molecular weight of 26,000–34,000. Both enzymes have a specifi activity two orders of magnitude higher than that of albumin, and both a inactivated by sulfhydryl-modifying reagents. A systematic survey of rat ti sues indicated that PGH to PGD isomerase activity is higher in brain and spin cord than in other tissues (67). PGD_2 is the principal cyclooxygenase product c rat and human mast cells (68).

PGD_2 inhibits platelet aggregation (69), increases platelet cAMP conten and has a platelet membrane receptor distinct from that for PGI_2 (70). I travenously infused PGD_2 is a peripheral vasodilator, pulmonary vasoconstri

tor, and bronchoconstrictor in the dog (71). Inhaled PGD_2 is a bronchoconstrictor in man (72).

The high concentrations of PGH to PGD isomerase in central nervous system suggest that PGD_2 may have neuromodulatory actions (67). PGD_2 augments cAMP content of cultured neuroblastoma cells (67) and induces depolarization of neuroblastoma-glioma hybrid cells (73). PGD_2 appears to decrease norepinephrine release from adrenergic nerve terminals in the cat (74). Instillation of PGD_2 into the third ventricle of rats increases slow-wave sleep (75).

PGD_2 intravenously infused into primates is metabolized to compounds reflecting various combinations of 11-keto reduction, dehydrogenation of the 15-hydroxyl, reduction of the delta^{13} double bond, β-oxidation, and ω-oxidation (76). Urinary excretion of markedly increased amounts of two metabolites of PGD_2 has been demonstrated in several patients with systemic mastocytosis, and mass spectrometric quantitation of one of these metabolites has been used as a diagnostic tool in this disorder (77, 78). Overproduction of PGD_2 may be involved in hypotensive episodes of patients with mastocytosis; chronic aspirin therapy reduces such attacks (77).

A novel circulating PGD_2 metabolite has recently been identified in plasma of a patient with mastocytosis and urine of a normal subject (79). This metabolite is a diastereoisomer of $PGF_{2\alpha}$, which has a beta configuration of the C-9 hydroxyl group and which is equipotent to $PGF_{2\alpha}$ as a pressor agent in the rat. The compound is formed from PGD_2 by an NADPH-requiring, cytosolic activity in liver.

Albumin catalyzes transformation of PGD_2 to 9-deoxy-delta^{9,12}-PGD_2, 15-deoxy-delta^{12,14}-PGD_2, and 9,15-dideoxy-delta^{9,12,14}-PGD_2 (80). In protein-free buffers, PGD_2 decomposes to several compounds including 9-deoxy-delta^9-PGD_2, and this compound suppresses the proliferation of cultured leukemia cells in vitro (81). Formation of this compound in vivo or by living cells has apparently not yet been demonstrated.

PROSTAGLANDIN E_2 Isomerization of PGH to PGE is catalyzed by a microsomal enzyme partially purified from sheep vesicular gland which exhibits a K_m of 10 μM for PGH_1 and a specific activity of 1–2 μmol PGE_2/min/mg protein (82). PGH to PGE isomerase activity from kidney is markedly stimulated by glutathione (83) and inactivated by sulfhydryl-modifying reagents (84).

As recently reviewed (85), PGE_2 is the predominant arachidonate metabolite from the kidney of many species, appears to antagonize ADH-induced water reabsorption (86, 87), and may participate in the modulation of renin release along with PGI_2 (88). PGE_2 is also produced by macrophages and may mediate some effects of macrophages on neighboring cells as well as influencing the functional state of the macrophage itself, as discussed in recent reviews (85, 89, 90). The possible participation of PGE_2 and other cyclooxygenase products in a variety of other processes has also recently been reviewed (91–94).

PRODUCTS OF THE 12-LIPOXYGENASE

The 12-Lipoxygenase Enzyme

Lipoxygenases catalyze incorporation of one oxygen molecule into polyunsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system to yield a 1-hydroperoxy-2,4-*trans*,*cis*-pentadiene product. The regional specificity of the lipoxygenase is designated by the number of the product carbon bearing the hydroperoxy group. The platelet 12-lipoxygenase converts arachidonate to 12-S-hydroperoxy-cicosa-5,8,10,14-tetraenoic acid (12-S-HPETE). The initial step in catalysis is stereospecific removal of the pro-S-hydrogen atom at C-10 with subsequent antarafacial insertion of molecular oxygen at C-12 (95).

Platelet 12-lipoxygenase activity is contained in both cytosolic and membrane fractions, and the cytosolic activity is an aggregated, lipid-containing form (96, 97). The kinetic properties, ionic sensitivity, and regional specificity of the cytosolic and membrane-associated activities are not distinguishable (98). The 12-lipoxygenase may therefore be a loosely membrane-associated enzyme and appears to be preferentially associated with internal platelet membranes rather than plasma or granule membranes (97, 98). Human platelet cytosolic 12-lipoxygenase activity elutes from size-exclusion columns in bands with approximate molecular weights of 100,000 and 160,000 and only the higher-molecular-weight species exhibits associated peroxidase activity (99) The 12-lipoxygenase activity from rat lung exhibits no peroxidase activity (100).

The platelet 12-lipoxygenase exhibits a kinetic lag period and is activated by trace concentrations of hydroperoxides (97, 101). Platelet 12-HETE synthesis continues for at least two hours after collagen stimulation, although TxB synthesis ceases after 3 minutes (101). The 12-lipoxygenase K_m for arachidonate is 5 μM for the rat lung enzyme (100) and 3.4 μM for the human platelet enzyme (97). Platelet 12-lipoxygenase has no known cofactor requirements and is fully active without exogenous calcium, although a rat basophilic leukemia cell 12-lipoxygenase is stimulated fivefold by 1 mM Ca^{2+} (102).

The platelet 12-lipoxygenase is not inhibited by nonsteroidal anti-inflammatory agents but is inhibited by ETYA, nordihydroguaiaretic acid (NDGA), BW755C, and a number of other compounds (97, 103, 104).

Metabolism of 12-HPETE

Reduction of 12-HPETE to its hydroxy analogue 12-HETE has been attributed to a peroxidase activity associated with the 12-lipoxygenase which is inhibited by nonsteroidal anti-inflammatory agents (99), but this observation needs substantiation. More recent evidence indicates that the selenium form of glutathione peroxidase plays a major role in reducing 12-HPETE to 12-HETE (105–107). Arachidonate stimulates platelet oxidation of [1-^{14}C]-glucose

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 33, Issue of August 13, pp. 23305–23310, 1999
Printed in U.S.A.

The Binding of Arachidonic Acid in the Cyclooxygenase Active Site of Mouse Prostaglandin Endoperoxide Synthase-2 (COX-2)

A PUTATIVE L-SHAPED BINDING CONFORMATION UTILIZING THE TOP CHANNEL REGION*

(Received for publication, May 14, 1999, and in revised form, June 8, 1999)

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The chemical mandates for arachidonic acid conversion to prostaglandin G_2 within the cyclooxygenase (COX) active site predict that the substrate will orient in a kinked or L-shaped conformation. Molecular modeling of arachidonic acid in sheep COX-1 confirms that this L-shaped conformation is possible, with the carboxylate moiety binding to Arg-120 and the ω-end positioned above Ser-530 in a region termed the top channel. Mutations of Gly-533 to valine or leucine in the top channel of mCOX-2 abolished the conversion of arachidonic acid to prostaglandin G_2, presumably because of a steric clash between the ω-end of the substrate and the introduced side chains. A smaller G533A mutant retained partial COX activity. The loss of COX activity with these mutants was not the result of reduced peroxidase activity, because the activity of all mutants was equivalent to the wild-type enzyme and the addition of exogenous peroxide did not restore full COX activity to any of the mutants. However, the Gly-533 mutants were able to oxidize the carbon 18 fatty acid substrates linolenic acid and stearidonic acid, which contain an allylic carbon at the ω-5 position. In contrast, linoleic acid, which is like arachidonic acid in that its most ω-proximal allylic carbon is at the ω-8 position, was not oxidized by the Gly-533 mutants. Finally, the ability of Gly-533 mutants to efficiently process ω-5 allylic substrates suggests that the top channel does not serve as a product exit route indicating that oxygenated substrate diffuses from the cyclooxygenase active site in a membrane proximal direction.

Prostaglandin endoperoxide synthase has two activities that are required for the production of PGH_2,[1] the essential precursor of prostaglandins, thromboxane, and prostacyclin (1). The first activity catalyzes the oxygenation and cyclization of arachidonic acid within the cyclooxygenase (COX) active site to produce PGG_2. PGG_2 then exits the cyclooxygenase active site and undergoes a two-electron reduction, yielding PGH_2 at the peroxidase active site (2). Two isoforms of COX exist, COX-1 and COX-2, that effect the same enzymatic reactions (3). They are approximately 60% identical in sequence and are highly homologous in both active site regions. Not surprisingly, their three-dimensional structures are nearly superimposable (4–6). COX-1 and COX-2 are mediators of numerous physiological and pathological responses, and therefore considerable effort has been devoted to developing selective COX inhibitors. This is especially true of COX-2, which is a significant contributor to inflammation, hyperalgesia, and cancer (7). Structural analysis of COX-inhibitor complexes has provided a detailed understanding of their interaction with the enzyme and insight into the mechanism of isoform selectivity (4–6, 8).

A less detailed picture is available for the interaction of the substrate arachidonic acid with the proteins. Crystal structures of COX-arachidonic acid complexes have not been reported, and so most of the available information has been developed by employing site-directed mutagenesis. It is generally agreed that Arg-120[2] ion pairs or hydrogen bonds to the carboxylate of the fatty acid (9–12) and that Tyr-385 removes the 13-*pro-S*-hydrogen in the first step of oxygenation (13, 14). However, the orientation of the rest of the substrate molecule, particularly the ω-end, is uncertain.

We have approached the problem of arachidonic acid-COX interaction by attempting to match the chemical mandates of the cyclooxygenase reaction to complementary regions on the protein. COX catalyzes the conversion of an achiral molecule into a product with five chiral centers (15). Noteworthy is the generation of the endoperoxide ring with the *trans*-substituted alkyl side chains. This stereochemistry is opposite that observed in the auto-oxidation of arachidonic acid in solution in which the alkyl side chains are oriented *cis* (16). Thus, the enzyme must orientate the arachidonic acid molecule in the COX active site to facilitate the formation of the *trans*-substituted ring. Any model for COX-arachidonate binding must accommodate this stereochemical mandate. Some time ago it was predicted that the enzyme holds arachidonate in a kinked or L-shaped conformation to facilitate cyclization to form a *trans*-disubstituted dioxabicycloheptane ring (17) (Fig. 1). Therefore, we modeled such a conformation into the COX-1 active site with the carboxylate adjacent to Arg-120 and the 13-*pro-S*-hydrogen adjacent to Tyr-385; this starting structure was then energy-minimized. The minimized conformation positioned the ω-end of arachidonate above Ser-530 projecting into a region that we term the top channel. The importance of

* This work was supported in part by Research Grant CA47479 and Center Grants ES00267 and CA68485 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of an Australian National Health and Medical Research Council C. J. Martin Fellowship.

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[1] The abbreviations used are: PG, prostaglandin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; COX, cyclooxygenase; mCOX-2, mouse cyclooxygenase-2; 11-(*R*)-HETE, 11-(*R*)-hydroxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid; 15-(*R*)-HETE, 15-(*R*)-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; PPIX, protoporphyrin IX.

[2] The numbering system used is in accord with that of COX-1.

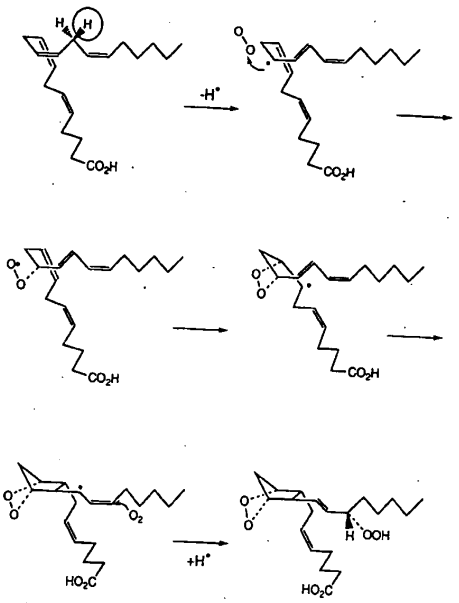

FIG. 1. **Mechanism of arachidonic acid conversion to PGG_2 by cyclooxygenase.** Adapted from Ref. 17.

the top channel was tested using site-directed mutagenesis of murine COX-2, which can be expressed at high levels in insect cells and purified to homogeneity. The results of the mutagenesis experiments are strongly supportive of a role for the top channel in binding the ω-end of arachidonic acid and are consistent with the predictions of the energy minimized model.

EXPERIMENTAL PROCEDURES

Materials—Linoleic acid (9*Z*,12*Z*-octadecadienoic acid), linolenic acid (9*Z*,12*Z*,15*Z*-octadecatrienoic acid), and stearidonic acid (6*Z*,9*Z*,12*Z*,15*Z*-octadecatetraenoic acid) were purchased from Cayman Chemical (Ann Arbor, MI). Arachidonic acid (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid) and [^{14}C]arachidonic acid were purchased from Nu-Check-Prep, Inc. and NEN Life Science Products, respectively. Electrophoresis and chromatography reagents were purchased from Bio-Rad Laboratories (Hercules, CA). All other reagents were purchased from Sigma unless otherwise stated.

Modeling—All modeling of arachidonic acid and sheep COX-1 was performed using InsightII (Biosyms Technologies, San Diego, CA) in the manner described previously (18). Arachidonic acid was built using the Builder module and then positioned within the cyclooxygenase active site with the carboxylate group in close proximity to Arg-120 and Tyr-355. The arachidonate main chain was then oriented upward toward the apex of the channel, with carbon 13 placed next to Tyr-385. The ω-end of arachidonate was then placed above Ser-530, protruding into the top channel toward Asn-375. Using the Discover module, several rounds of energy minimization were performed, maintaining the sheep COX-1 structure as a fixed entity and allowing arachidonate to freely rotate into its most energetically favorable orientation. Several rounds of energy minimization were necessary to obtain a structure that was energetically favorable and consistent with the predicted stereochemical requirements of PGG_2 production, *i.e.* maintenance of all *cis* double bonds and positioning of the 13-*pro-S*-hydrogen in close proximity to Tyr-385.

Mutant Construction—Site-directed mutagenesis was performed on a mCOX-2 pBS(+) vector (Stratagene, La Jolla, CA) using the Quick Change site-directed mutagenesis kit (Stratagene). Mutant containing regions were subcloned into the mCOX-2 pVL1393 baculovirus expression vector (PharMingen, San Diego, CA) using the *Stu*I restriction site in mCOX-2 and the *Xba*I restriction site present in both the pBS(+) and pVL1393 vectors. The subcloned region was fully sequenced to ensure that no accidental mutations were incorporated.

Protein Expression and Purification—Wild-type and mutant protein was expressed by homologous recombination of the mCOX-2·pVL1393 vector with the Baculogold vector (PharMingen) in SF-9 cells (Novagen, Madison, WI). After virus amplification, 4 liters of SF-9 cells (95–100% viable) were grown in TNM-FH medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% L-glutamine, and 0.1% (v/v) pluronic F68 and then infected with fresh viral stock. Upon reaching 65–70% viability, the 4-liter total volume was harvested by centrifugation at 2500 rpm in a Sorvall RC-3B, and the pellet was washed in ice-cold phosphate-buffered saline and recentrifuged. The final cell pellet was stored at −70 °C.

Purification of wild-type and mutant COXs were performed at 4 °C in a manner similar to that described previously (19). Frozen cells were resuspended to 30 × 10^6 cells/ml in 80 mM Tris-HCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1 mM diethyldithiocarbamic acid, pH 7.2. After centrifugation at 100,000 × *g* for 45 min, the pellet was resuspended using a Dounce homogenizer to a final volume of 72 ml. Solubilization of the COX protein from the membrane was initiated by the dropwise addition of 8 ml of 11% (w/v) CHAPS. After stirring for 1 h, the sample was recentrifuged as described above and the supernatant removed and then diluted 4-fold by the addition of 20 mM Tris-HCl, 0.4% CHAPS, 0.1 mM EDTA, and 0.1 mM diethyldithiocarbamic acid, pH 8.0 (Buffer B). The diluted sample was then loaded onto a 25-ml Macro-prep High-Q ion exchange column equilibrated with Buffer B. COX enzyme was eluted with a linear gradient (500 ml) of increasing KCl to 0.3 M. An analytical 7.5% SDS-polyacrylamide gel electrophoresis was run of candidate COX-containing fractions to determine the fractions to pool for the gel filtration procedure. Appropriate tubes were concentrated in an Amicon concentrator (Amicon, Beverly, MA) to a final volume of 1.5 ml. The sample was then loaded onto a 90-ml Superose-12 column that was pre-equilibrated with 20 mM Tris-HCl, 0.4% CHAPS, 0.15 M NaCl, pH 8.0. Fractions containing COX enzyme, as determined from SDS-polyacrylamide gel electrophoresis analysis (as described above), were concentrated to approximately 2 mg/ml and stored at −70 °C. The purity of wild-type and mutant COX-2 proteins was evaluated by analysis of a Coomassie-stained 7.5% SDS-polyacrylamide gel using an E-C Apparatus Model EC910 scanning densitometer.

Cyclooxygenase Activity—Oxygen consumption was measured with a Gilson Model 5/6 oxygraph (Gilson Medical Electronics, Inc., Middletown, WI) fitted with a Clark electrode and a thermostatted cuvette set to 37 °C in a 100 mM Tris-HCl, 500 μM phenol, pH 8.0, buffer. The rate and magnitude of oxygen uptake were determined in the manner previously described (20).

Peroxidase Assays—The peroxidase activity of all purified proteins was measured using the guaiacol peroxidase assay. Purified wild-type mCOX-2 or mCOX-2 mutants were suspended in 1 ml of 118 mM Tris-HCl, pH 8.0, at a final concentration of 100 nM. Guaiacol at 500 μM was added, and the dual cuvettes were auto-zeroed in a Shidmadzu UV 160U spectrophotometer before the addition of 400 μM H_2O_2 to the same cuvette. Oxidation of guaiacol was monitored at 436 nm (E_{436} = 6390 M^{-1} cm^{-1}), and the initial rate was measured from the linear portion of the curve.

Cyclooxygenase Product Assays—Cyclooxygenase product assays were performed with purified protein reconstituted with 2 eq of hematin. Reactions were initiated by the addition of 100 μM [^{14}C]arachidonic acid. Conditions such as the concentration of phenol, supplementation with H_2O_2, and time were varied and are indicated in the figure captions. All assays were terminated and analyzed by thin layer chromatography in the manner described previously (21).

RESULTS

Modeling—Arachidonate was anchored at the carboxyl end through interactions with Arg-120 and Tyr-355 and by positioning the 13-*pro-S*-hydrogen adjacent to Tyr-385. The ω-end was placed into the region we term the top channel. Several rounds of energy minimization and refinement were carried out to optimally position the substrate in the sheep COX-1 active site. In the final model (Fig. 2), the carboxylic acid moieties of arachidonic acid are 3.0 and 3.1 Å from Arg-120 and Tyr-355, respectively, and the 13-*pro-S*-hydrogen is 2.4 Å from the hydroxyl of Tyr-385. The ω-end of arachidonate protrudes into the top channel region, and although the opening to this channel is narrow, there is sufficient room for carbons 17–20 to reside above Ser-530 and Leu-534 and project into the solvent-accessible cavity toward Gly-533. Carbon 20 resides 3.3 Å from the α carbon of Gly-533.

Mutant COX-2 Characterization—To test this hypothesis, we constructed a series of site-directed mutants at Gly-533. This

FIG. 2. **Stereo image of the modeled structure of the sheep COX-1·arachidonic acid complex.** The carboxyl end of arachidonic acid (oxygens in *red*, sp3 carbons in *blue*, and sp2 carbons in *green*) is bound to Arg-120 (*yellow*) and Tyr-355 (*green*). The archidonate 13-*pro-S*-hydrogen (*white*) on carbon 13 (yellow) is 2.1 Å from Tyr-385 (*orange*), and the 13-*pro-R*-hydrogen (*pink*) is 4.1 Å from Tyr-385. Other residues shown are Leu-352 in *dark blue*, Trp-387 in *white*, Ile-523 in *brown*, Ser-530 in *red*, Gly-533 in *pink*, and Leu-534 in *gray*. The distances from arachidonate to other regions of the protein include the following: arachidonate C-6 to Ile-523 O, 3.4 Å; C-10 to Trp-387 Cη, 2.8 Å; C-12 to Ile-352 Cδ², 3.4 Å; C-15 to Ser-530 Oγ, 3.5 Å; C-18 to Gly-533 Cα, 5.9 Å; and C-19 to Gly-533 Cα, 4.7 Å.

residue is conserved in all COX sequences, and we anticipated that increasing the steric bulk at this position would reduce arachidonate binding. Both wild-type and mutant COX-2 cDNAs were expressed in insect cells from baculovirus vectors, and recombinant proteins were purified by ion exchange and gel filtration chromatography as described under "Experimental Procedures." All of the purified proteins were shown by densitometric scanning of a 7.5% SDS-polyacrylamide gel to be equal to or greater than 80% pure (Table I).

Table I lists both the peroxidase activity and the cyclooxygenase activity of the three Gly-533 mutants. The similarities of the peroxidase activities to that of wild-type COX-2 demonstrate that the mutations did not introduce gross structural perturbations. However, the cyclooxygenase activities of all three mutants were affected significantly. The G533L and G533V mutants were unable to convert arachidonate to PGG_2, whereas the G533A mutant had a much slower initial rate for substrate conversion and demonstrated just 26% of wild-type total COX-2 activity.

One possibility that could explain the low turnover rate observed with G533A could be a decreased activation of cyclooxygenase catalysis. PGG_2 released from the cyclooxygenase active site is converted to PGH_2 at the peroxidase active site with concomitant formation of a heme-oxo complex (22). This complex oxidizes Tyr-385 and generates the catalytically active tyrosyl radical. As the G533A substitution compromises conversion of arachidonate to PGG_2, this lower activity could be further pronounced because of less PGG_2 available to maximally activate all the COX molecules in solution. To address the concern of insufficient peroxide activation, three different approaches were employed: 1) lowering the concentration of reducing substrate (phenol) in the assay buffer to slow down the reduction of exogenous peroxides and cyclooxygenase-synthesized fatty acid hydroperoxides; (2) adding H_2O_2 to generate higher oxidation states of the peroxidase; and (3) adding enzyme-synthesized fatty acid hydroperoxide as an activator.

Reducing the phenol concentration from 500 to 100 μM resulted in an increase in cyclooxygenase activity of the G533A mutant from 5.5 to 18.9% converted substrate (Fig. 3*A*). Likewise, the addition of 15 μM H_2O_2 increased the conversion of arachidonate from 3.4 to 22% (Fig. 3*B*). However, changing these conditions did not restore cyclooxygenase activity to the level observed with an equivalent amount of wild-type mCOX-2 nor did it restore any activity to either G533V or G533L. To evaluate whether PGG_2 could enhance G533A turnover, G533A mCOX-2 was incubated with a 13-fold lower concentration of Mn-PPIX reconstituted ovine COX-1. Mn-PPIX reconstituted ovine COX-1 has full cyclooxygenase activity but only 0.8% of the peroxidase activity of Fe-PPIX reconstituted COX-1 (23, 24). Thus, G533A mCOX-2 should be able to use the COX-1-derived PGG_2 to increase the rate of tyrosyl radical formation and cyclooxygenase activity. After subtracting the cyclooxygenase activity resulting from COX-1 turnover, it was found that the G533A activity was equivalent to that observed when G533A was incubated with 15 μM H_2O_2 as an activator.[3] These results suggest that the reduced cyclooxygenase activity of the G533A mutant leads to a slower rate of auto-activation. However, even when maximally activated, the cyclooxygenase activity of this mutant is reduced by 80% compared with the wild-type enzyme, and the G533V and G533L mutants are completely inactive.

Analysis of the arachidonate/sheep COX-1 model suggests that the most likely reason for the reduced activity is steric hindrance between arachidonate carbon 20 and the introduced side chains at position 533. Therefore, the Gly-533 mutants were compared with wild-type enzyme for their ability to metabolize two fatty acids that are substrates for COX-2 but contain an abstractable hydrogen closer to the ω-end (ω-5 po-

TABLE I
Characterization of Gly-533 mutant cyclooxygenases

The purity of wild-type and mutant COX-2 proteins was determined from Coomassie-stained 7.5% SDS-polyacrylamide gels. Peroxidase activities were analyzed using the guaiacol peroxidase assay, and the cyclooxygenase activity initial rates and total product production were determined in the manner described under "Experimental Procedures." Values are the average of three determinations ± S.E. wt, wild type.

Construct	Purity	Peroxidase activity	Cyclooxygenase activity, initial rate	Cyclooxygenase activity, total products
	%	*% wt mCOX-2*	*% wt mCOX-2*	*% wt mCOX-2*
wt mCOX-2	84.3	100	100	100
G533A	80.0	99 ± 5	7.6 ± 0.5	26.3 ± 0
G533V	80.0	97 ± 4	0	0
G533L	80.0	107 ± 3	0	0

[3] B. C. Crews, unpublished observation.

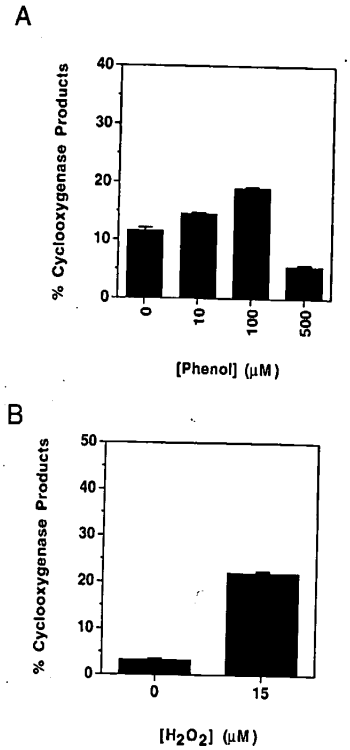

FIG. 3. **Increase in cyclooxygenase activity of G533A mCOX-2 by increasing steady-state peroxide levels.** *A*, reduction in the phenol concentration from 500 to 10 μM; or *B*, the addition of 15 μM H_2O_2. For *A*, G533A (44 nM) was incubated in 100 mM Tris-HCl, pH 8, with the indicated concentration of phenol and 50 μM [^{14}C]arachidonic acid for 5 min at 37 °C; and for *B*, G533A (44 nM) was incubated with 5 μM [^{14}C]arachidonic acid in 100 mM Tris-HCl, pH 8, containing 500 μM phenol for 60 s at 37 °C. For both *A* and *B*, the reactions were acid-extracted with cold termination solution, run on silica thin-layer chromatographic plates, and scanned for radioactive products in the manner indicated under "Experimental Procedures." Each data point is the average of three determinations ± S.E.

sition). It was predicted that these shorter substrates (Fig. 4) would not protrude as far into the top channel, thereby reducing the chances of steric clash with the introduced side chain at position 533. To obtain maximal rates and extents of O_2 uptake, this study was first performed in the presence of 15 μM H_2O_2 (Table II). All three Gly-533 mutants were able to oxidize linolenic and stearidonic acid. In fact, G533A was more efficient than wild-type at converting linolenic acid, and both G533A and G533V metabolized more linolenic acid on a molar basis than did the wild-type enzyme. With stearidonic acid, the rate of substrate conversion for G533A was similar to wild-type mCOX-2, but it decreased upon increasing the chain length at position 533 to valine and leucine. As seen with linolenic acid, the total turnover was greater with G533A and G533V than with wild-type. As a control, incubations were performed with linoleic acid, which contains two fewer carbons at the carboxylate end of the substrate but the same number of carbons as arachidonate between its abstractable hydrogen at its ω-end. None of the Gly-533 mutants oxidized linoleic acid.

To establish whether exogenously added peroxide was necessary to obtain maximal cyclooxygenase activity with linolenic and stearidonic acid,[4] assays were performed in the absence of

[4] Results virtually identical to those seen in Table III with linolenic acid were obtained with stearidonic acid.

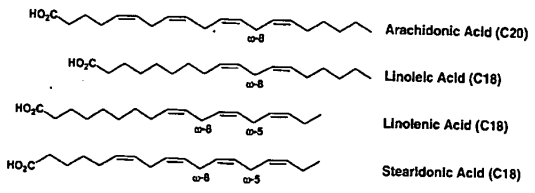

FIG. 4. **Structures of fatty acid substrates of COX-2.**

H_2O_2 but were then supplemented with H_2O_2 after 2 min to complete the reaction (Tables III). Comparison of wild-type mCOX-2 treated with or without 15 μM H_2O_2 (Tables II and III) showed that the addition of H_2O_2 had little effect on either the rate or the total substrate conversion. The Gly-533 mutants, in contrast, showed much more dependence on H_2O_2, as both the rate and amount of substrate converted were reduced in its absence. The addition of 15 μM H_2O_2 to the reaction was able to reactivate substrate conversion for the Gly-533 mutants.

DISCUSSION

We propose a model for the binding of arachidonic acid in the cyclooxygenase active site that is consistent with the chemical mandates for its oxidation to PGG_2 and with existing information on its interaction with individual protein residues. The carboxylate of arachidonate is anchored to Arg-120 and Tyr-355 by ionic and hydrogen bonding interactions, respectively (Fig. 2). The substrate backbone then projects upward into the apex of the cyclooxygenase active site where it bends around the 9,10-single bond into an L-shape. Carbon 13 is positioned under Tyr-385 with the 13-*pro-S*-hydrogen 2.4 Å from the phenolic hydroxyl group. Finally, the ω-end of arachidonate extends above Ser-530 and Leu-534 into the top channel. Maintenance of this L-shaped conformation is essential for generating the dioxobicycloheptane ring of PGG_2 with the pendant alkyl chains oriented *trans* to each other. Considering the chemical steps necessary to generate PGG_2 from the perspective of this structural model suggests that the individual reactions can occur with minimal motion of the bound intermediates. Furthermore, the model predicts the generation of PGG_2 with all five stereocenters in the correct absolute configuration, because once the arachidonate is bound as indicated in Fig. 2, the only available space through which O_2 can approach the radical intermediates is through the center of the active site channel. Thus, O_2 approaches the bound fatty acid from the opposite side from which the 13-*pro-S*-hydrogen is removed. This antarafacial relationship is consistent with the stereochemistry of PGG_2.

The key feature of our model is that the ω-end of arachidonate projects into an area of the protein we term the top channel, which is located above Ser-530 and Leu-534. There are two major pieces of experimental information consistent with this hypothesis. The first is that introduction of steric bulk at position 533 by site-directed mutagenesis significantly reduces or completely abolishes the ability of the mutant COX-2s to oxidize arachidonic acid. The loss of oxygenase activity is not due to a major structural change in the proteins, because the peroxidase activity of each of the position 533 mutants is identical to that of wild-type enzyme. Likewise, the loss of activity is not due to an inability of the mutant proteins to activate the cyclooxygenase activity, although activity is stimulated to some extent by addition of H_2O_2. The second piece of experimental information consistent with a role for the top channel in arachidonate binding is that all of the position 533 mutants are able to oxidize unsaturated fatty acid substrates that contain three less carbons at their ω-end than arachidonate relative to the position of the hydrogen abstracted by the tyrosyl radical of

TABLE II

Activity of H_2O_2-activated wild-type and Gly-533 mutants with different fatty acid substrates

Oxygen consumption was measured in the manner described under "Experimental Procedures." Fatty acids (100 μM) and H_2O_2 (15 μM) were added to the oxygraph cell followed by hematin-reconstituted enzyme (150 nM). Values are the average of three determinations ± S.E.

	mCOX-2	G533A	G533V	G533L
μmol arachidonic acid/min/mg	9.6 ± 0.2	0.8 ± 0.1	0	0
μM O_2 consumed	50.7 ± 0.2	15.3 ± 0.2	0	0
μmol linoleic acid/min/mg	5.3 ± 0.6	0	0	0
μM O_2 consumed	16.7 ± 0.6	0	0	0
μmol linolenic acid/min/mg	5.0 ± 0.6	6.7 ± 0.6	2.9 ± 0.2	1.0 ± 0.1
μM O_2 consumed	11.7 ± 0.2	25.0 ± 0.9	19.7 ± 0.2	5.3 ± 0.16
μmol stearidonic acid/min/mg	6.6 ± 0.3	6.2 ± 0.1	2.7 ± 0.1	1.2 ± 0.1
μM O_2 consumed	15.0 ± 0.5	25.0 ± 0.5	21.2 ± 0.3	10.3 ± 0.2

TABLE III

Activity of wild-type and Gly-533 mutants with linolenic acid before and after H_2O_2 addition

Oxygen consumption was measured as described under "Experimental Procedures." Linolenic acid (100 μM) and hematin-reconstituted enzyme (150 nM) were added to the oxygraph cell, and oxygen consumption was monitored for 2 min before the addition of H_2O_2 (15 μM). The values in the "Oxygen consumption after H_2O_2" column represent the total oxygen consumption by the enzyme. Values are the average of three determinations ± S.E.

Mutant	Oxygen consumption with linolenic acid	Rate with linolenic acid	Oxygen consumption after H_2O_2	Rate after H_2O_2
	μM	μmol/min/mg	μM	μmol linolenic acid/min/mg
Wild type	12.9 ± 0.2	6.1 ± 0.3	0	0
G533A	5.8 ± 0.4	1.9 ± 0.2	16.0 ± 0.6	1.8 ± 0.3
G533V	2.5 ± 0	1.0 ± 0.2	18.7 ± 0.3	2.9 ± 0.1
G533L	0	0	6.8 ± 0.3	0.6 ± 0.1

Tyr-385. By contrast, the mutants are unable to oxidize linoleic acid, which contains two fewer carbons on the carboxyl end than arachidonate but the same distance between its abstractable hydrogen and its ω-end.

The ability of the Gly-533 mutants to oxidize linolenic and stearidonic acids but not arachidonic or linoleic acids establishes that the distance between the hydrogen abstracted by the tyrosyl radical of Tyr-385 and the ω-end of the fatty acid is an important determinant of COX-fatty acid interactions. Positioning of fatty acids in the top channel may be as important for determining substrate specificity as the positioning of the carboxylate adjacent to Arg-120 at the mouth of the channel. There appears to be flexibility in the distance from the hydrogen abstracted by Tyr-385 to the carboxylate of the fatty acid, especially in the case of COX-2. Substrates with an abstractable hydrogen at the 11, 13, or 14 carbon from the carboxylate are oxidized by COX-2. Laneuville *et al.* (25) have proposed that the substrate adapts to the accessible space between Tyr-385 and Arg-120 by adopting a linear conformation (11-carbon length) or a kinked conformation (13- or 14-carbon lengths). The extra space in the side pocket off the main channel of COX-2 relative to COX-1 may permit the kinking required to accommodate different length substrates.

A corollary of the ability of the position 533 mutants to oxidize linolenic and stearidonic acid is that the top channel does not represent part of an exit route to the top surface of the protein. An examination of the crystal structures of both COX-1 and COX-2 indicates that the channel around Gly-533 connects to another channel that eventually leads to an opening on the surface of the protein near the dimer interface (6). Conceivably, this opening could represent an exit port through which product escapes the cyclooxygenase active site. Indeed, this could be an explanation for the inability of sterically blocked mutants (*e.g.* G533V and G533L) to oxidize arachidonic acid. The first molecules of PGG_2 synthesized from arachidonic acid would be unable to exit and would prevent the binding of additional molecules of arachidonic acid into the cyclooxygenase active site. However, blocking the exit channel should also prevent the release of the products of linolenic acid and stearidonic acid, thereby stopping turnover. The high activity of the Gly-533 mutants toward these carbon 18 fatty acids eliminates the possibility that the top channel is part of an exit route for products.

Our model is consistent with the chemistry of the production of PGG_2 from arachidonic acid and with all the currently available site-directed mutagenesis results. Thus, it is likely that this is the conformation by which COX enzymes convert arachidonic acid to its major enzymatic product. This conclusion does not rule out the possibility that arachidonate binds in the cyclooxygenase active site in alternate conformations. For example, arachidonate is oxygenated to a series of hydroxy acids by cyclooxygenase including 11-(*S*)-HETE, 15-(*S*)-HETE, and 15-(*R*)-HETE (15, 26). The production of 11-(*S*)-HETE and 15-(*S*)-HETE have been assumed to result from O_2 trapping of carbon radicals produced following removal of the 13-*pro-S*-hydrogen. However, 15-(*R*)-HETE is produced by aspirin-acetylated COX-2 and is likely to arise from an alternate conformation of arachidonic acid (26). Indeed, Xiao *et al.* (27) have recently shown that 15-(*R*)-HETE also can be made by unacetylated COX-2 at high concentrations of arachidonic acid. Thus, 15-(*R*)-HETE appears to represent an alternate product that results from a less favored substrate conformation.

Acknowledgments—We thank K. Kozak and D. Goodwin for helpful discussions and suggestions.

REFERENCES

1. Ohki, S., Ogino, N., Yamamoto, S., and Hayaishi, O. (1979) *J. Biol. Chem.* **254**, 829–836
2. Smith, W. L., and Marnett, L. J. (1991) *Biochim. Biophys. Acta* **1083**, 1–17
3. Herschman, H. R. (1996) *Biochim. Biophys. Acta.* **1299**, 125–140
4. Picot, D., Loll, P. J., and Garavito, R. M. (1994) *Nature* **367**, 243–249
5. Luong, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M. F. (1996) *Nat. Struct. Biol.* **3**, 927–933
6. Kurumbail, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1996) *Nature* **384**, 644–648
7. DuBois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van de Putte, B. A., and Lipsky, P. E. (1998) *FASEB J.* **12**, 1063–1073
8. Loll, P. J., Picot, D., Ekabo, O., and Garavito, R. M. (1996) *Biochemistry* **35**, 7330–7340
9. Picot, D., and Garavito, R. M. (1994) *FEBS Letts.* **346**, 21–25
10. Mancini, J. A., Riendeau, D., Falgueyret, J.-P., Vickers, P. J., and O'Neill, G. P. (1995) *J. Biol. Chem.* **270**, 29372–29377
11. Bhattacharyya, D. K., Lecomte, M., Rieke, C. J., Garavito, R. M., and Smith, W. L. (1996) *J. Biol. Chem.* **271**, 2179–2184
12. Rieke, C. J., Mulichak, A. M., Garavito, R. M., and Smith, W. L. (1999) *J. Biol. Chem.* **274**, 17109–17114
13. Shimokawa, T., Kulmacz, R. J., DeWitt, D. L., and Smith, W. L. (1990) *J. Biol. Chem.* **265**, 20073–20076
14. Goodwin, D. C., Gunther, M. H., Hsi, L. H., Crews, B. C., Eling, T. E., Mason, R. P., and Marnett, L. J. (1998) *J. Biol. Chem.* **273**, 8903–8909
15. Hamberg, M., and Samuelsson, B. (1967) *J. Biol. Chem.* **242**, 5336–5343
16. O'Connor, D. E., Mihelich, E. D., and Coleman, M. C. (1984) *J. Am. Chem. Soc.* **106**, 3577–3584
17. Marnett, L. J., and Maddipati, K. R. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., and Grisham, M. B., eds) Vol. 1, pp. 293–334, CRC Press, Boca Raton, FL
18. Lanzo, C. A., Beechem, J., Talley, J., and Marnett, L. J. (1998) *Biochemistry*

37, 217–226
19. Gierse, J. K., McDonald, J. J., Hauser, S. D., Rangwala, S. H., Koboldt, C. M., and Seibert, K. (1996) *J. Biol. Chem.* **271**, 15810–15814
20. Kalgutkar, A. S., Crews, B. C., and Marnett, L. J. (1996) *Biochemistry* **35**, 9076–9082
21. Kalgutkar, A. S., Crews, B. C., Rowlinson, S. W., Garner, C., Seibert, K., and Marnett, L. J. (1998) *Science* **280**, 1268–1270
22. Dietz, R., Nastainczyk, W., and Ruf, H. H. (1988) *Eur. J. Biochem.* **171**, 321–328
23. Odenwaller, R., Maddipati, K. R., and Marnett, L. J. (1992) *J. Biol. Chem.* **267**, 13863–13869
24. Strieder, S., Schaible, K., Scherer, H.-J., Dietz, R., and Ruf, H. H. (1992) *J. Biol. Chem.* **267**, 13870–13878
25. Laneuville, O., Breuer, D. K., Xu, N., Huang, Z. H., Gage, D. A., Watson, J. T., Lagarde, M., DeWitt, D. L., and Smith, W. L. (1995) *J. Biol. Chem.* **270**, 19330–19336
26. Lecomte, M., Laneuville, O., Ji, C., DeWitt, D. L., and Smith, W. L. (1994) *J. Biol. Chem.* **269**, 13207–13215
27. Xiao, G., Tsai, A.-L., Palmer, G., Boyar, W. C., Marshall, P. J., and Kulmacz, R. J. (1997) *Biochemistry* **36**, 1836–1845

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INSTRUCTIONS FOR LETTERS TO THE EDITOR

Editorial comment in the form of a Letter to the Editor is invited; however, it should not exceed 800 words, with a maximum of 10 references and no more than 2 figures or tables and no subdivision for an Abstract, Methods, or Results. Letters should have no more than 3 authors. Full name(s) and address of the author(s) should accompany the letter as well as the telephone number and fax number (if available). Financial associations or other possible conflicts of interest should always be disclosed. To expedite receipt of letters, we encourage authors outside Canada to communicate by fax (416-967-7556).

Roles of COX-1 and COX-2

To the Editor:

Much attention is currently devoted to the emerging generation of nonsteroidal antiinflammatory drugs (NSAID) that selectively inhibit the cyclooxygenase (COX) activity of the inducible isozyme of prostaglandin endoperoxide (PGH) synthase, known generally as COX-2. In reviewing this exciting area of research, Hawker[1] and de Brum-Fernandes[2] elegantly remind us that while the notion of selectivity of inhibition of COX-2 is highly seductive, there remains considerable uncertainty in defining the potential disadvantages that may be inherent with prolonged COX-2 inhibition. Moreover, a role of COX-1 (the "constitutive" isozyme) in inflammation cannot be excluded[1,3], and special attention must be paid to the possible roles of COX-2 in physiological processes[2,3]. Indeed, numerous issues will have to be addressed when COX-2 specific agents are submitted for clinical assay[3]. However, we should not forget that additionally, these new NSAID will inherit persisting unresolved issues that relate to currently available agents; we must be careful not to carry these forward with these new compounds!

First, the current practice of differentiating NSAID through nomenclature is unacceptable. The names of drugs are often formed by combining syllables taken from chemical sources to produce chemical sounding names. For example, *aza, prop,* and *one* are all well known syllables combined in *azapropazone* to produce what is really a nonsense word. To supplement such names, it is common practice to refer to subsections of the entire structure. Thus, indomethacin has been classified as a 3-indoylacetic acid, and more broadly as an arylacetic acid. There are no rules about such choices except that (where possible) the classifier is meant to indicate the part(s) of the structure believed to influence the pharmacological activity. Thus, indomethacin is not classified (as it certainly could be) as a derivative of 4-chlorobenzamide or of *p*-anisidine merely because there is no current interest in those substructures. Difficulties, however, arise when novelty is sought. Thus, for example, the reclassification of etodolac from an *indole* to a *pyranocarboxylate*, while confusing, also conveys erroneous impressions when interpreted systematically. It is important that modern systematic nomenclature is concerned only with formal structural relations. Consequently, it should be noted that systematic nomenclature, excellent for its own purposes when used correctly, cannot automatically provide good classifications for subsequent differentiation in clinical practice. That is, the presence in a structure of specified structural segments cannot be used to imply that the compound has certain chemical or pharmacological properties.

Issues of biodisposition remain unresolved. Although NSAID readily enter the central nervous system (CNS) in humans, and for some members of this class, at levels that exceed the level of free drug in plasma[4], the relative contribution of CNS effects to pain relief is unknown[4]. Moreover, we have very little understanding of the mechanism(s) of transfer of NSAID into the CNS, which may possibly be controlled by active processes, and not simply by passive diffusion[4]. Similarly, it is not at all clear why parenteral administration of NSAID should produce gastric toxicity, since transfer of NSAID in clinical use (i.e., acidic) from plasma into gastric cells apparently cannot take place according to classical theory of absorption and partitioning[5–7]. However, accumulation of NSAID within gastric mucosal cells may be accommodated by such theory by using a new model for the transfer of weakly acidic (pK_a 3–5) xenobiotic drugs[7]. Interestingly, this new model predicts that regardless of the degree of selectivity for inhibition of COX-2 (the target for preferential inhibitors), even the weakest inhibitors of COX-1 may accumulate within gastric mucosal cells to levels where damage may result.

Possible paradoxical situations arise over the use of NSAID as we discover more about the role of cytokines in the expression of chronic inflammatory disorders. For example, Kumkumian and co-workers[8] investigated the effects of platelet derived growth factor (PDGF) and interleukin 1 (IL-1) upon the regulation of synoviocytes from patients with rheumatoid arthritis (RA). They observed that IL-1 inhibited PDGF-stimulated synoviocyte proliferation, and that exogenous PGE_2, a prostaglandin (PG) known to be produced in response to IL-1, dramatically inhibited synoviocyte proliferation induced by PDGF. Importantly, when these same cells were treated with PDGF and IL-1 in the presence of indomethacin, whereas the DNA synthetic response to IL-1 was unaffected, PDGF and IL-1 operated synergistically to stimulate synoviocyte proliferation. These results were confirmed by other workers (see McCormack 1997[9] for review). Using NIH-3T3 fibroblasts, aspirin pretreatment increased both the rate and amplitude of PDGF-stimulated PGH synthase mRNA induction. Taken together, these results suggest that PG negatively modulate PDGF-mediated effects upon fibroblast/fibroblast-like synthetic activity. Moreover, PG, notably PGD_2, PGE_2, and PGI_2, also inhibit the release of PDGF from platelets. Thus, given that both the platelet and PDGF have recently been shown to play a key role in the hyperplastic response of synovial connective tissue cells in RA, then intervention by NSAID apparently poses a dilemma. That is, whereas inhibition of PG synthesis likely diminishes pain through an attenuation of peripheral sensitization, paradoxically this may result in disinhibition of both PDGF release, and PDGF-mediated synoviocyte proliferation and PGH synthase mRNA induction. Faced with this possible therapeutic dilemma, it will be interesting to observe progress with the novel NSAID lornoxicam (launched 1997), which, in addition to potently inhibiting COX activity[9], has also been shown to dramatically inhibit the release of PDGF from human platelets *in vitro*[9]. Importantly, this dose related effect takes place at levels of lornoxicam that are entirely consistent with levels of free drug in the synovial fluid of chronically inflamed joints of patients with either RA or osteoarthritis, following single or repeat dosing. The launch of this new NSAID serves nicely to illustrate an important point. That is, COX is but one part of the inflammatory process. We should view COX-2 selective agents as a step forward and not as an alternative generic!

Despite a plèthora of available NSAID, no robust evidence has yet been presented in support of the notion that these compounds do not represent a single generic. Using metaanalysis, attempts to differentiate individual NSAID as analgesics for the management of acute inflammatory pain have been hindered by considerable bias and confounding[4,10–13]. A different situation may exist, however, with multiple dosing schedules of NSAID in the management of chronic inflammatory pain. Longterm use of these compounds may provide an opportunity for distinguishing those compounds that have effects upon pain/inflammatory mediators, in addition to the ubiquitous inhibition of COX activity[9].

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REFERENCES

1. Hawker G. Prescribing nonsteroidal antiinflammatory drugs — what's new? J Rheumatol 1997;24:243–5.
2. de Brum-Fernandes AJ. New perspectives for nonsteroidal antiinflammatory therapy. J Rheumatol 1997;24:246–8.
3. Pairet M, Engelhardt G. Distinct isoforms (COX-1 and COX-2) of cyclooxygenase: possible physiological and therapeutic implications. Fundam Clin Pharmacol 1996;10:1–15.
4. McCormack K. Non-steroidal anti-inflammatory drugs and spinal nociceptive processing. Pain 1994;59:9–43.
5. McCormack K, Brune K. Classical absorption theory and the development of gastric mucosal damage associated with the nonsteroidal antiinflammatory drugs. Arch Toxicol 1987;60:261–9.
6. McCormack K, Brune K. The amphiprotic character of azapropazone and its relevance to the gastric mucosa. Arch Toxicol 1990;64:1–6.
7. McCormack K. Mathematical model for assessing risk of gastrointestinal reactions to NSAIDs. In: Rainsford KD, editor. Azapropazone. 20 years of clinical use. Lancaster, UK: Kluwer Academic Publishers; 1989;81–93.
8. Kumkumian GK, Lafyatis R, Remmers EF, Case JP, Kim SJ, Wilder RL. Platelet-derived growth factor and IL-1 interactions in rheumatoid arthritis. Regulation of synoviocyte proliferation, prostaglandin production, and collagenase transcription. J Immunol 1989;143:833–7.
9. McCormack K. Some new insights into the pharmacology of NSAIDs. In: Sawynok J, Cowan A, editors. Novel aspects of pain management. Opioids and beyond. New York: John Wiley & Sons Inc.; 1997:in press.
10. McCormack K, Brune K. Dissociation between the antinociceptive and the antiinflammatory effects of the nonsteroidal antiinflammatory drugs. Drugs 1991;41:533–47.
11. McCormack K, Brune K. Towards defining the analgesic role of nonsteroidal antiinflammatory drugs in the management of acute soft tissue injuries. Clin J Sport Med 1993;3:106–17.
12. McCormack K. The spinal actions of NSAIDs and the dissociation between antiinflammatory and analgesic effects. Drugs 1994;47:28–45.
13. McCormack K, Urquhart E. Correlation between nonsteroidal anti-inflammatory drug efficacy in a clinical pain model and the dissociation of their anti-inflammatory and analgesic properties in animal models. Clin Drug Invest 1995;9:88–97.

Dr. de Brum-Fernandes replies

To the Editor:

At the dawn of a new generation of nonsteroidal antiinflammatory drugs (NSAID) Dr. McCormack highlights some important, yet unresolved issues on the pharmacology of these drugs, and I would like to comment on some of the issues he raised. One can only agree that the present classification of NSAID is confused, often incorrect from a chemical point of view and, most important, that grouping according to chemical characteristics does not always imply that similar compounds have common pharmacological properties. As has often been proposed, classifications based on pharmacological characteristics such as half-life or reversibility of cyclooxygenase inhibition would be more appropriate since they convey important information to clinicians. I also agree that most names of drugs are nonsense words. On a practical basis, however, I believe this to be acceptable. As a clinician, I would not be comfortable prescribing 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid (indomethacin) several times a day. A very important point that was not approached by Dr. McCormack is how to classify these drugs according to their selectivity towards COX-1 and COX-2. Several questions remain unanswered, such as how specific should a molecule be to offer therapeutical and pharmacological advantages? From a theoretical point of view the IC_{50}s for the isoenzymes should be as far as to allow complete inhibition of COX-2 without significantly affecting COX-1 activity, implying at least a 100-fold difference. Although that should be a good starting point in the laboratory, it is difficult to predict how each isoenzyme will be inhibited *in vivo* if one takes into account characteristics of drug and isoenzyme distribution. No consensus exists even on how to get to this starting point in the laboratory. As we recently pointed out[1], several details can affect the results obtained from in vitro cyclooxygenase inhibition assays; different results can be obtained with purified enzymes versus whole, living cells, or animal versus human isoenzymes; some molecules are time dependent inhibitors and act differently if the inhibition is instantaneous or preceded by incubation with the drug; the availability of the cyclooxygenase substrate, arachidonic acid, is controlled in some assays but not in others; reversibility of the inhibition, which can have important therapeutical consequences, is not always tested. The lack of a standard for cyclooxygenase inhibition assays severely hampers the comparison between different molecules as well as their classification, and an effort should be made by the scientific community and the pharmaceutical industries to settle this issue.

Dr. McCormack also comments on paradoxical situations that may arise with the widespread inhibition of cyclooxygenases. Paradoxes about prostaglandin actions have long been known but never completely understood. In bone, for example, PGE_2 can induce bone formation in low and stimulate bone resorption in high concentrations[2]. These situations reflect our incomplete understanding of the fine mechanisms implicated in the physiological and pharmacological actions of prostaglandins. These autacoids act through interaction with specific membrane receptors, and possibly with some intracellular receptors[3]. At least one type of receptor has been described for each natural prostaglandin (for review see Coleman, *et al* [4]). One of these subtypes presents 3 to 7 subtypes generated by alternative splicing, depending on the species. Thus, up to 14 different prostaglandin receptors mediating different and even opposed biological effects may theoretically exist in a given biological system. In such a context it is not difficult to understand why the widespread inhibition of prostaglandin synthesis by NSAID may lead to paradoxical situations where the net gain, in terms of a therapeutical or pharmacological effect, may be close to none. It is probable that most of these paradoxes will be explained as the distribution of prostaglandin receptors and the characterization of the biological effects mediated by them on different tissues and cells become known. This will open the way to more specific and target-restricted pharmacological interventions with the use of specific prostaglandin agonists and antagonists. It is possible to imagine, for example, specific antagonists that will block the effect of prostaglandins on nociception or vascular permeability without affecting the receptors mediating neutrophil immobilization or inhibition of PDGF-induced synoviocyte proliferation. COX-2 specific inhibitors are today's holy grail, but intervention aimed at specific prostaglandin receptors will probably be the next generation of antiinflammatory drugs. Moreover, given the ubiquitous nature of prostaglandins, knowledge on receptor distribution will have implications not only for the field of inflammation but also on other areas where prostaglandins have important biological effects.

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REFERENCES

1. Lora M, Denault JB, Leduc R, de Brum-Fernandes AJ. Systematic pharmacological approach to the characterization of NSAIDS. Prostaglandins Leukot Essent Fatty Acids 1998;59:55–62.
2. Raisz LG. The role of prostaglandins in the local regulation of bone metabolism. Progr Clin Biol Res 1990;332:195–203.
3. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-deoxy-delta(12,14)-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR-gamma. Cell 1995;83:803–12.

4. Coleman R, Smith WL, Narumiya S. VIII. International Union of Pharmacology Classification of Prostanoid Receptors: properties, distribution, and structure of the receptor and their subtypes. Pharmacol Rev 1994;46:205–29.

Association Between Acquired Free Protein S Deficiency, Anticardiolipin Antibodies, and Thrombotic Events in Rheumatoid Arthritis

To the Editor:

We read with great interest the article by Tomás, *et al* concerning the relationship between different natural anticoagulant proteins, antiphospholipid antibodies (aPL), and thrombotic events in systemic lupus erythematosus (SLE)[1]. The authors suggest an association between aPL positivity and reduced free protein S levels in these patients. Recent reports have confirmed increased concentrations of aPL [e.g., anticardiolipin antibodies (aCL)] in patients with rheumatoid arthritis (RA), resulting in an increased risk of different vascular diseases including arterial or venous thromboses and vasculitis[2,3]. In contrast, however, is the recent description of low levels of free protein S in some patients with aPL suggesting these antibodies may be involved in mechanisms causing increased risk of thrombosis[4].

In the light of these observations, we would introduce our experience concerning the association of altered free protein S levels, positivity of aCL, and thrombotic complications in patients with RA.

We evaluated levels of total and free protein S, protein C, and antithrombin III in 102 seropositive (IgM rheumatoid factor, IgM-RF) female patients with RA (mean age 53 ± 10 yrs) who were not taking steroids, methotrexate, azathioprine, cyclophosphamide, hydroxychloroquine, or other drugs interfering with the hemostatic variables. Seventy-four healthy age matched female volunteers (mean age 53 ± 8 yrs) were evaluated as controls.

Protein C and antithrombin III concentrations were measured by chromogenic substrates (Kabi; Vitrum, Stockholm, Sweden) and total and free protein S by an ELISA (Diagnostica Stago, Asnières, France). Patients in the study satisfied the 1987 American Rheumatism Association criteria for RA and were treated in our RA clinic, specializing in extraarticular involvement, a secondary referral center from Genova and the surrounding region.

Patients enrolled had frequent RA complications, such as hypertension, venous and arterial thrombosis, and cardiovascular diseases. Patients were grouped as "aCL positive" (n = 35; mean age 53 ± 11 yrs) and "aCL negative" (n = 67, mean age 54 ± 9 yrs) depending on aCL positivity. Serum IgG and IGM aCL were assayed by ELISA, as described[5].

The combined group of patients with RA was found to have a significantly higher rate of venous and/or arterial thrombosis compared to controls [n = 22/102 (22%) vs 3/74 (4%), respectively; p = 0.001]. In particular, thrombotic events were diagnosed in 34% of aCL positive patients (n = 12/35, 7 venous, 5 arterial thrombosis), in 15% of aCL negative patients (n = 10/67, all venous thrombosis), and in only 4% of controls (n = 3/74, all venous thrombosis). Controls with thrombosis had had recent articular trauma.

Almost all the patients showed significantly (p = 0.01) lower free protein S levels (mean 86 ± 21%, range 48–130) compared to controls (mean 109 ± 23%, range 60–150). Patients, both aCL positive and negative, showed lower total protein S (mean 99 ± 12%, range 74–130) and protein C levels (mean 102 ± 23%, range 70–132) and higher antithrombin III concentrations (mean 101 ± 28%, range 78–122) than controls (100 ± 21%, range 82–130, for protein S, 109 ± 23%, range 60–150 for protein C, and 100 ± 16%, range 80–120 for antithrombin III). However, the differences were not statistically significant.

The reduction of free protein S levels in aCL positive patients with RA compared to both aCL negative patients and controls was statistically significant (mean 66 ± 12%, range 48–88 vs 97 ± 16%, range 65–130 and 109 ± 23%, range 60–150, respectively; p = 0.001). No differences in total protein S, protein C, and antithrombin III were detected among the groups.

When we analyzed the patients with a history of thrombotic events, significantly lower levels of free protein S were found in aCL positive patients compared to aCL negative patients and controls (p = 0.001).

Patients with RA with positive aCL and history of arterial and/or venous thromboses showed lower levels of free protein S compared to patients with positive aCL without history of thrombosis (p = 0.003) (see Table 1). Interestingly, free protein S levels in aCL positive patients with RA with arterial thrombosis were slightly lower compared to patients with venous thrombosis (55 ± 4 vs 58 ± 9%); however, the difference was not statistically significant.

Further, the comparison of the mean IgG aCL levels between patients with RA, with and without thromboses was significantly different (47 ± 3 vs 31 ± 2 GPL units, respectively; p = 0.01). On the other hand, the difference in IgM aCL concentrations between the 2 groups of patients was not significant (10 ± 2 vs 8 ± 2 MPL units, respectively; p = 0.35). In particular, IgG and IgM titers in aCL positive patients with arterial thrombosis were significantly elevated compared to those with venous thrombosis (58 ± 3 GPL units and 13 ± 4 MPL units vs 39 ± 2 GPL units and 8 ± 1 MPL units, respectively; p = 0.05).

Our results seem to agree with previous observations showing an

Table 1. Free and total protein S, protein C, and antithrombin III values in aCL positive and negative patients with RA and controls with regard to their history of thrombosis. Values are mean ± SEM (range).

	RA				Controls	
	aCL Positive		aCL Negative			
	With Thrombosis (n = 12)	Without Thrombosis (n = 23)	With Thrombosis (n = 10)	Without Thrombosis (n = 57)	With Thrombosis (n = 3)	Without Thrombosis (n = 71)
Free Protein S (% of normal)	57 ± 2*† (48–76)	70 ± 2 (50–88)	101 ± 7 (68–128)	96 ± 2 (65–130)	127 ± 7 (120–140)	109 ± 3 (60–150)
Total Protein S (% of normal)	91 ± 4 (74–128)	103 ± 3 (89–128)	103 ± 4 (80–130)	99 ± 1 (80–130)	107 ± 7 (100–120)	98 ± 4 (90–120)
Protein C (% of normal)	101 ± 4 (78–124)	102 ± 3 (70–120)	118 ± 4 (97–130)	101 ± 2 (70–132)	112 ± 8 (95–120)	104 ± 2 (80–140)
Antithrombin III (% of normal)	96 ± 3 (78–114)	100 ± 3 (79–118)	100 ± 6 (80–120)	103 ± 3 (82–122)	101 ± 7 (80–116)	102 ± 3 (82–120)

*p = 0.003, aCL positive patients with RA with thrombosis vs no thrombosis; †p = 0.001, aCL positive patients with RA with thrombosis vs both aCL negative RA patients and controls with thrombosis.